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Anti-obesity effects of narirutin and hesperidin in immature *Citrus reticulata* extract via AMPK activation in high-fat diet-fed mice

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ABSTRACT

Obesity, a major global health concern, increases the risk of various diseases. *Citrus reticulata*, commonly used in traditional medicine, contains bioactive compounds that have potential metabolic benefits. The aim of the study was to assess the effects of immature *C. reticulata* extract (ICRE) and its key bioactive compounds, narirutin and hesperidin, in a mouse model of obesity. Six-week-old C57BL/6N mice were fed either a normal or high-fat diet for 12 weeks and treated with 100, 150, or 200 mg/kg ICRE or 2 mg/kg narirutin and hesperidin. Body weight, adipocyte size, serum lipase activity, and serum levels of triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and blood glucose were measured. Leptin and leptin receptor mRNA expression levels were analyzed in adipose tissue, along with mRNA and protein expression levels of AMP-activated protein kinase (AMPK) pathway-related molecules and markers of fatty acid oxidation and lipid synthesis. ICRE significantly reduced body weight and adipocyte size, improved lipid profiles, and decreased leptin levels and lipase activity in mice receiving a high-fat diet. It also enhanced AMPK activation, promoted fatty acid oxidation, and inhibited lipid synthesis. These effects were more pronounced than those observed with narirutin and hesperidin. ICRE demonstrated superior efficacy compared with narirutin and hesperidin by more effectively regulating lipid metabolism and energy expenditure through the AMPK pathway, leading to greater reductions in fat accumulation and improved metabolic outcomes. These findings suggest that ICRE is a promising natural therapeutic agent for obesity management.

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1. Introduction

Obesity is characterized by excessive fat accumulation, which can cause physiological alterations that contribute to the development of non-communicable diseases such as type 2 diabetes, cardiovascular disorders, and cancer^[1-4]. It is now a global public health concern affecting increasing numbers of individuals. According to the

most recent global analysis by the NCD Risk Factor Collaboration published in *The Lancet* in 2024^[5], the number of adults with obesity increased significantly from 1990 to 2022 in 188 countries for women and in nearly all countries for men. As of 2022, an estimated 879 million adults and 159 million children and adolescents were classified as obese. Obesity is now more prevalent than underweight in the majority of countries, especially in North America, the Middle East, and island nations in Polynesia and Micronesia, highlighting a global shift towards an obesity-dominant burden of malnutrition. The striking upward trend in obesity demonstrates the need for effective and sustainable interventions to manage obesity and its associated complications.

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Conventional weight management strategies, such as calorie restriction and exercise, have yielded limited long-term success. Adherence to strict dietary and physical activity regimens is challenging, particularly in environments that promote overeating and sedentary behavior. Pharmacological interventions, such as orlistat and semaglutide, are often associated with substantial adverse effects, including gastrointestinal disturbances, cardiovascular risks, and psychiatric symptoms, limiting their acceptability and long-term use^[6]. Consequently, there is an urgent need for novel, safe, and effective approaches to obesity management.

Functional foods and natural products are being increasingly investigated for their potential to combat obesity. Plant-derived compounds typically possess a safer profile than synthetic drugs, making them an appealing option as long-term use is required to manage obesity^[7]. Functional foods, particularly those containing the plant-based compounds polyphenols and flavonoids, have been shown to exert anti-inflammatory, antioxidant, and lipid-modulating effects^[8–10]. Flavonoids, a subclass of polyphenols, are bioactive plant compounds widely distributed in fruits, vegetables, tea, and wine. They can be classified into several groups, including flavanones, flavonols, flavones, isoflavones, anthocyanins, and flavan-3-ols, which exhibit distinct biological activities^[11–14]. Among these, flavanones such as narirutin and hesperidin are predominantly found in citrus fruits and are known for their strong metabolic regulatory effects. The bioactive components found in citrus fruits, particularly flavonoids, have shown promise in the treatment of metabolic disorders^[12,15–16]. In particular, citrus-derived flavonoids have been investigated for their ability to modulate obesity-related metabolic pathways, with effects including AMP-activated protein kinase (AMPK) activation, peroxisome proliferator-activated receptor gamma (PPAR γ) inhibition, and suppression of adipocyte differentiation. These compounds also help mitigate oxidative stress and inflammation, both of which are key contributors to metabolic dysfunction in obesity^[17–20].

Citrus reticulata, commonly known as mandarin or immature citrus, has garnered considerable attention, particularly in East Asia, for its traditional medicinal uses^[21]. The fruit is rich in bioactive flavonoids, with narirutin and hesperidin being the most abundant constituents, and *C. reticulata* extract has emerged as a potential agent for promoting weight loss and improving metabolic health. Hesperidin, a glycosylated form of hesperetin, has been shown to regulate lipid metabolism by inhibiting adipogenesis, reducing triglyceride accumulation, and enhancing mitochondrial function in adipocytes^[12,22–23]. It also plays a role in improving insulin sensitivity and glucose homeostasis. Meanwhile, narirutin is known to activate AMPK, a key regulator of energy metabolism, thereby promoting fatty acid oxidation and inhibiting lipogenesis, which helps prevent excessive fat accumulation^[15–16,22]. Studies conducted on animal models have demonstrated the efficacy of citrus-derived flavonoids in reducing fat accumulation, improving lipid profiles, and alleviating metabolic dysfunction^[24]. Notably, these flavonoids are present in higher concentrations in immature fruits than in their fully ripened counterparts, potentially enhancing their pharmacological efficacy^[25–27], and immature citrus has been shown to be more effective in reducing fat accumulation and enhancing lipid metabolism^[15]. However, there is a need to better understand the precise mechanisms through which immature *C. reticulata* extract (ICRE) and its bioactive components, such as narirutin and hesperidin, exert their anti-obesity effects.

Adipocyte differentiation, which leads to fat accumulation, is regulated by a complex interplay of transcription factors. CCAAT/enhancer-binding protein (C/EBP) β and C/EBP δ mediate the expression of PPAR γ and C/EBP α , both of which are abundantly expressed in mature adipocytes^[28–31]. PPAR γ is crucial for adipocyte differentiation, regulating the transcription of multiple genes involved in fat accumulation. Additionally, AMPK is a vital metabolic regulator that responds sensitively to metabolic stress^[18,32]. AMPK activation inhibits adipocyte differentiation, reduces fatty acid synthesis, and promotes fatty acid oxidation, thereby restoring energy homeostasis. The activation of AMPK by flavonoids such as hesperidin and narirutin suggests a potential mechanism through which their anti-obesity effects are mediated^[33–35].

In this study, we aimed to explore the anti-obesity potential of ICRE in comparison to a 1:1 mixture of its key bioactive compounds, narirutin and hesperidin (Nar/Hes), to determine whether the extract provides synergistic benefits that exceed the effects of the individual components. We also aimed to clarify the molecular mechanisms through which ICRE modulates adipocyte differentiation. This study will contribute to the development of natural, plant-based therapies for obesity management and highlight the potential of functional foods as viable therapeutic options.

2. Material and methods

2.1 Materials

ICRE was supplied by Novarex Co., Ltd. (Cheongju, Korea). Acetonitrile was purchased from Duksan Pure Chemicals Co., Ltd. (Ansan, Korea) and 95% food-grade ethanol was obtained from Daehan Ethanol Life Co., Ltd. (Hwaseong, Korea). Narirutin was purchased from ChemFaces (CFN99543; Wuhan, China) and hesperidin was purchased from Sigma Aldrich (PHR1794; St. Louis, MO, USA).

2.2 Plant material and sample preparation

The plant material, *C. reticulata* (Blanco), was authenticated and taxonomically identified by Biomedic Co., Ltd. (Bucheon, Korea). Genomic DNA was extracted from 3 randomly selected fruit and peel samples to confirm species identity using nuclear (internal transcribed spacer) and chloroplast (*PsbK-PsbI*, *trnH-psbA*, *ycf1*, *rbcL*) barcoding markers for bidirectional sequencing. The species identity was confirmed through DNA barcoding techniques and cross-referenced with the NCBI GenBank database (www.ncbi.nlm.nih.gov). A voucher specimen of the authenticated plant material has been deposited at NOVAREX Co., Ltd. (Cheongju, Korea) for future reference (voucher number NR-31-13-RM). The raw materials used in the production of ICRE were harvested between mid-August and mid-September 2023 and purchased from the Jeju Plant Resource Research Institute (Jeju, Korea). Samples were prepared by NOVAREX Co., Ltd. Specifically, 2 kg dried immature *C. reticulata* was used for extraction, which was performed using food-grade 95% ethanol diluted 1:1 (*m/m*) with tap water at (70 $\pm$ 5) °C under reflux for 3 h. The filtered extract was concentrated using a vacuum rotary evaporator and dried into powder. Longitudinal analysis confirmed that the citrus fruits belonged to the species *C. reticulata*.

2.3 Concentration of narirutin and hesperidin in ICRE

The narirutin and hesperidin content of ICRE was quantified using high performance liquid chromatography (HPLC) and a UV detector (1260 Infinity; Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a C₁₈ reversed-phase HPLC column (J'sphere ODS-H80; 4.6 mm × 250 mm, 4 μm; YMC Co., Ltd., Kyoto, Japan) at 25 °C, with mobile phases of 0.1% phosphoric acid in acetonitrile (A) and 0.1% phosphoric acid in water (B) applied at a flow rate of 1.0 mL/min. The injection volume was 10 μL and the detection was recorded at a wavelength of 280 nm. The gradient program was as follows: 0–20 min, 82% B; 20–60 min, 82%–45% B; and 60–75 min, 45%–82% B.

2.4 Animal experiments

Six-week-old male C57BL/6N mice were purchased from Hyochang Science (Daegu, Korea) and housed in temperature-controlled ((22 ± 2) °C) and humidity-controlled ((55 ± 5)%) rooms under a 12 h light/dark cycle. All experimental protocols were approved by the local ethics committee (PNU-2023-0300). After 1 week of acclimatization, mice were randomly divided into groups. The control group received a 10% fat diet and the remaining mice received a 60% high-fat diet (HFD; DooYeol Biotech, Seoul, Korea) for 12 weeks. Mice in the HFD group (*n* = 40) were randomly divided into 5 groups (*n* = 8 per group) and received a HFD containing either no supplementation, ICRE (100, 150, or 200 mg/kg), or a 1:1 ratio of narirutin/hesperidin (Nar/Hes; 2 mg/kg). Diet and water intake were monitored twice a week.

2.5 Oral glucose tolerance test

After 12 weeks of feeding, an oral glucose tolerance test (OGTT) was performed on mice following a 6 h fast. A glucose solution (2 g/kg body weight) was administered by oral gavage. Blood glucose levels were measured in blood from the tail vein using a glucometer (Accu-Chek; Roche Diagnostics, Indianapolis, IN, USA) 0, 15, 30, 60, and 120 min after glucose administration. The area under the curve of glucose levels over time was calculated to assess glucose tolerance, using a formula described previously^[36].

2.6 Serum analysis

Blood samples were collected from the abdominal aorta of each mouse and placed into Microtainer Blood Collection Tubes (BD, Franklin Lakes, NJ, USA). These were centrifuged at 12 000 r/min for 20 min at 4 °C to separate the serum, which was then stored at −80 °C for subsequent biochemical analyses. Serum levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using an automated chemistry analyzer (Prestige 24i; Tokyo Boeki Medisys Inc., Tokyo, Japan)^[37].

2.7 Lipase activity assay

Blood samples were collected from the abdominal aorta of each mouse and centrifuged at 13 000 r/min for 20 min to obtain serum. Pancreatic lipase was dissolved in cold distilled water at 50 mg/mL

and centrifuged at 4 000 r/min at 4 °C to collect the supernatant, 50 μL of which was added to 200 μL Tris buffer (0.1 mol/L, pH 8.8). After incubating the mixture at 37 °C for 15 min with shaking, 5 mmol/L 4-nitrophenyl octanoate in dimethyl sulfoxide was added, and the reaction was further incubated at 37 °C for 30 min. Absorbance was measured at a wavelength of 412 nm using a microplate reader.

2.8 Histopathological analysis

Epididymal adipose tissue was embedded in paraffin and cut into 5-μm sections. The sections were deparaffinized in xylene, rehydrated through a series of graded alcohols (100%, 95%, 70%, and 50%), and rinsed with distilled water. The sections were stained with Harris hematoxylin solution for 5 min and placed in running tap water for 5 min to develop the color. Then, sections were stained with eosin solution for 2 min, followed by dehydration through graded alcohols (70%, 95%, and 100%) and clearing in xylene. Finally, the sections were mounted using a resinous medium and examined under a light microscope (Olympus CX41RF; Olympus Co., Tokyo, Japan).

2.9 Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from epididymal adipose tissue using a GeneAll RiboEx Total RNA extraction reagent (GeneAll Biotechnology, Seoul, Korea) and Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). The isolated RNA was reverse transcribed into complementary DNA (cDNA) using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Real-time polymerase chain reaction (qRT-PCR) was conducted using a SensiFAST™ SYBR® No-ROX kit (Bioline, London, UK) and a CFX Connect™ Real-Time PCR system (Bio-Rad Laboratories, Inc.). The primer sequences used are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as the internal control and was used to normalize mRNA levels.

Table 1
List of murine primers used for qRT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>SREBP-1</i>	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
<i>CEBP</i>	TGGACAAGAACAGCAACGAGTAC	GCAGTTGCCCATGGCCTTGAC
<i>PPAR_γ</i>	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
<i>FAS</i>	GGCTCTATGGATTACCCAAGC	CCAGTGTTCGTTCTCTCGGA
<i>ACC</i>	GGAGATGTACGCTGACCGAGAA	ACCCGACGCATGGTTTTC
<i>ACL</i>	TGGATGCCACAGCTGACTAC	GGTTCAGCAAGGTCAGCTTC
<i>SCD1</i>	CCTACGACAAGAACATTCAATCCC	CAGGAACTCAGAAGCCCCAAGC
<i>ATGL</i>	CAACGCCACTCACATCTACGG	GGACACCTCAATAATGTTGGCAC
<i>HSL</i>	CCGTTCTGCAGACTCTCTC	CCACGCAACTCTGGGTCTAT
<i>PPAR_α</i>	TGTCGAATATGTGGGGACAA	AATCTTGACGCTCCGATCAC
<i>CPT1</i>	GGCATAAACGCAGAGCATTCCTG	CAGTGTCCATCCTCTGAGTAGC
<i>UCP</i>	TGGCCTCTCCAGTGGATGTG	CGTGGTCTCCAGCATAGAAG
<i>Leptin</i>	TGAGTTTGTCGAAGATGGACC	GCCATCCAGGCTCTCTGG
<i>Leptin receptor</i>	TGATGTGTCAGAAATTCTATGTGGT	AGCAACAGTGAAGACTGTTTGG
<i>GAPDH</i>	GTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA

2.10 Western blotting analysis

Epididymal adipose tissue was treated with ProEX CETi lysis buffer (TransLab Biosciences, Daejeon, Korea) and the resulting lysate was centrifuged at 12 000 r/min for 20 min at 4 °C. The total protein concentration of the supernatant was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% powdered skimmed milk at room temperature for 1 h and subsequently incubated overnight at 4 °C with primary antibodies against phosphorylated (p)-AMPK, AMPK, PPAR γ , and p-ATP-citrate lyase (ACL) (Cell Signaling Technology, Inc., Danvers, MA, USA); sterol regulatory element-binding protein 1 (SREBP-1), fatty acid synthase (FAS), and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and ACL, acetyl-CoA carboxylase (ACC), and p-ACC (Abclonal, Wuhan, China) at dilutions ranging from 1:2 000 to 1:5 000. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and antigen-antibody complexes were visualized using an EZ-Western Lumi Pico Alpha detection kit (Dogen Bio, Seoul, Korea).

2.11 Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by Tukey's honest significant difference (HSD) post hoc test was used to compare multiple groups. Differences were considered statistically significant at $P < 0.05$. Statistical significance among groups is indicated by different letters, where groups sharing the same letter are not significantly different.

3. Results

3.1 Concentrations of narirutin and hesperidin in ICRE

Narirutin (Fig. 1A) and hesperidin (Fig. 1B) are the predominant flavonoids in citrus fruits. HPLC confirmed that ICRE, prepared from plant material using 50% ethanol as described in Section 2.2. Plant material and sample preparation, had an extraction yield of 48.5% and contained (12.6 ± 2.5) mg/g narirutin and (11.7 ± 2.3) mg/g hesperidin. The retention times of narirutin and hesperidin in standard solutions were 21.220 and 27.870 min, respectively (Fig. 1C).

In ICRE, the corresponding retention times were 21.062 and 27.778 min (Fig. 1D). The equal and elevated levels of both flavonoids in ICRE in the present study suggest they may have therapeutically relevant bioactive potential. In comparison, previous studies have reported narirutin and hesperidin levels in citrus fruits to be (4.52 ± 0.31) and (9.14 ± 0.32) mg/g, respectively^[15]. These concentrations of narirutin and hesperidin in ICRE suggest a therapeutically relevant bioactive potential, comparable to those found in citrus fruits. The balanced and elevated levels of both flavonoids (narirutin at (12.6 ± 2.5) mg/g and hesperidin at (11.7 ± 2.3) mg/g) may contribute to the observed metabolic health benefits, consistent with the known properties of citrus bioflavonoids in modulating lipid metabolism and reducing oxidative stress.

3.2 Effects of ICRE on body weight in HFD-fed mice

HFD group mice exhibited significantly greater weight gain than that of control group mice, confirming the successful induction of obesity. Mice receiving an HFD supplemented with ICRE or Nar/Hes gained less body weight than that gained by mice receiving an HFD only (Fig. 2A). Among the treatments, 200 mg/kg ICRE demonstrated the most substantial and statistically significant reduction in weight gain (Fig. 2B). These results suggest that the bioactive compounds in ICRE, including narirutin and hesperidin, act synergistically to produce more potent effects than Nar/Hes, and that ICRE potentially contains additional phytochemicals that enhance its efficacy. Hematoxylin and eosin staining revealed notable histological differences between the groups. Mice fed an HFD alone exhibited enlarged, hypertrophic adipocytes with reduced inter-adipocyte spacing, indicative of excessive fat accumulation (Fig. 2C). In contrast, mice treated with ICRE or Nar/Hes displayed significantly smaller adipocytes with increased inter-adipocyte space, suggesting reduced lipid accumulation and adipocyte hypertrophy. Among the groups, the 200 mg/kg ICRE group showed the most pronounced reduction in adipocyte size compared to the HFD group, closely resembling that of the control group. Measurement of adipocyte size indicated a significant increase in the HFD group compared with the control group, confirming hypertrophic expansion of adipocytes. Compared to the HFD group, all groups receiving ICRE exhibited a marked reduction in adipocyte size, with the 200 mg/kg ICRE group showing the most pronounced decrease (Fig. 2D). Adipocytes in the HFD group were 6-fold larger than those in the control group; ICRE treatment reduced this by 75%, demonstrating a greater effect than Nar/Hes treatment, which resulted in a 50% reduction. These results underscore the superior efficacy of ICRE in mitigating HFD-induced adipocyte hypertrophy and lipid accumulation, supporting its potential anti-obesity effects.

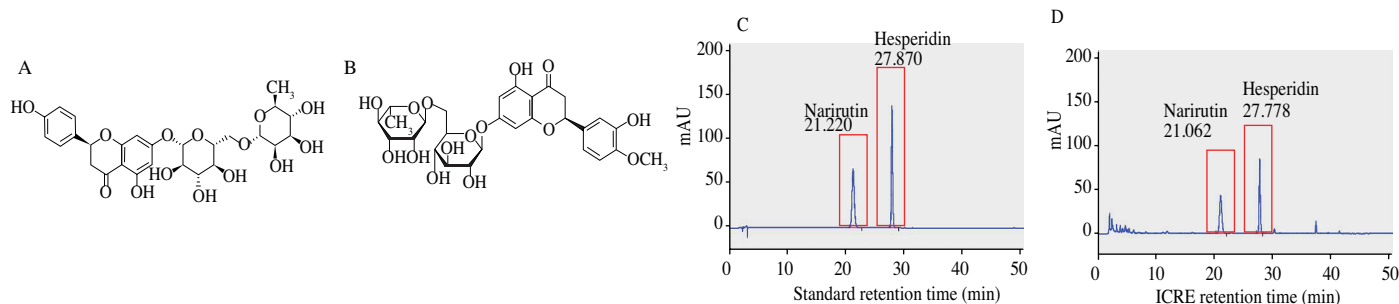


Fig. 1 Analysis of narirutin and hesperidin in ICRE. Structural formula of (A) narirutin and (B) hesperidin. Chromatograms showing the presence of narirutin and hesperidin in (C) the standard solution and (D) ICRE.

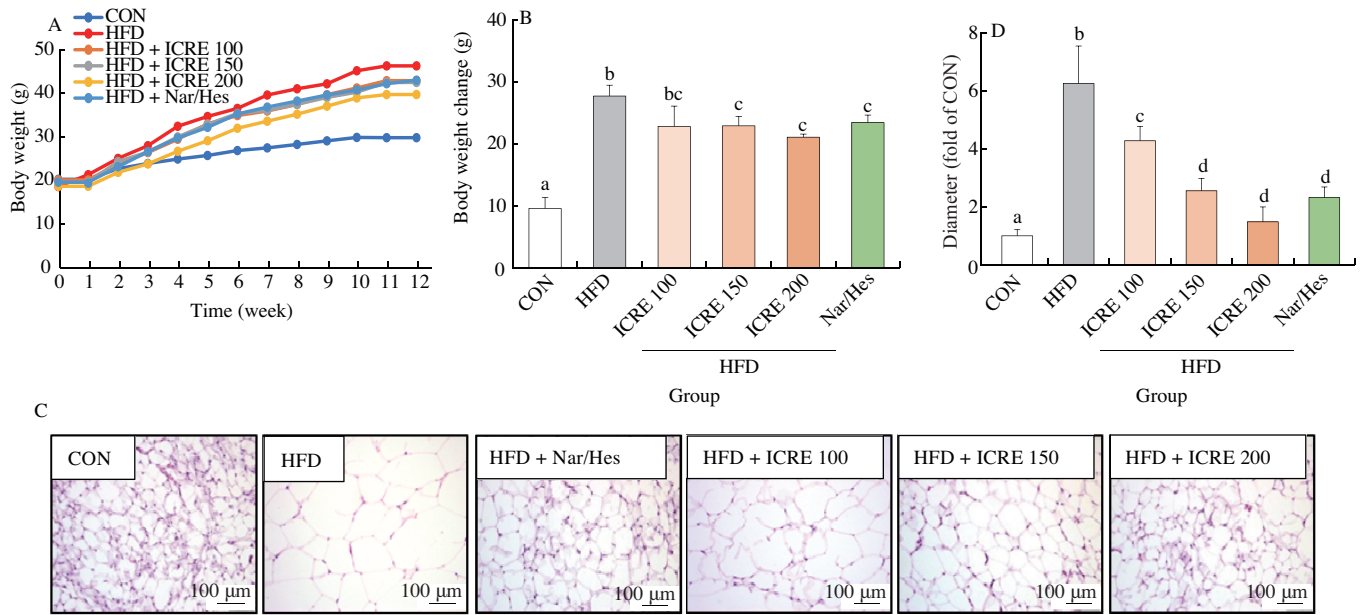


Fig. 2 Effects of ICRE and Nar/Hes on body weight and epididymal fat in HFD-fed mice. (A) Body weight of mice receiving different diets over the 12-week experimental period. (B) Body weight increase over the 12-week experimental period. (C) Representative images of hematoxylin and eosin-stained epididymal adipose tissues. (D) Quantitative assessment of changes in epididymal adipose tissue area. For multiple group comparisons, different letters (a, b, c, d) indicate significant differences among groups ($P < 0.05$, Tukey's honest significant difference test). CON, control. The same below.

3.3 Effects of ICRE on lipid profiles and glucose metabolism in HFD-fed mice

Serum analysis revealed that mice in the HFD group exhibited significantly elevated levels of TG, TC, HDL-C, and LDL-C compared with those in control group mice (Figs. 3A-D). Treatment with ICRE or Nar/Hes significantly inhibited these increases. Significant reductions in these parameters were observed even in the 100 mg/kg ICRE group, indicating the effectiveness of ICRE at lower doses. To evaluate the impact of ICRE and Nar/Hes on glucose metabolism, an OGTT was performed (Figs. 3E and F). Mice in the HFD group exhibited elevated glucose levels, indicative of impaired glucose regulation due to diet-induced obesity. However, mice treated with ICRE demonstrated a dose-dependent reduction in glucose levels compared with those in HFD group mice. Treatment with Nar/Hes also inhibited the increase in glucose levels. In addition, serum ALT and AST levels were significantly increased in the HFD

group compared to the control group, reflecting hepatic dysfunction associated with diet-induced obesity (Figs. 3G and H). Treatment with ICRE significantly reduced ALT levels in a dose-dependent manner, suggesting a protective effect against HFD-induced liver damage. The reduction in ALT and AST levels was more pronounced in the ICRE-treated groups than in the Nar/Hes group, further supporting the superior efficacy of ICRE in preserving hepatic function. These findings suggest that ICRE is effective in reversing the detrimental effects of a HFD on lipid profiles and glucose metabolism, and superior to Nar/Hes.

3.4 Effects of ICRE on leptin levels and lipase activity in HFD-fed mice

Leptin, a critical adipokine, plays a significant role in regulating energy balance by signaling satiety and controlling fat storage^[38-39].

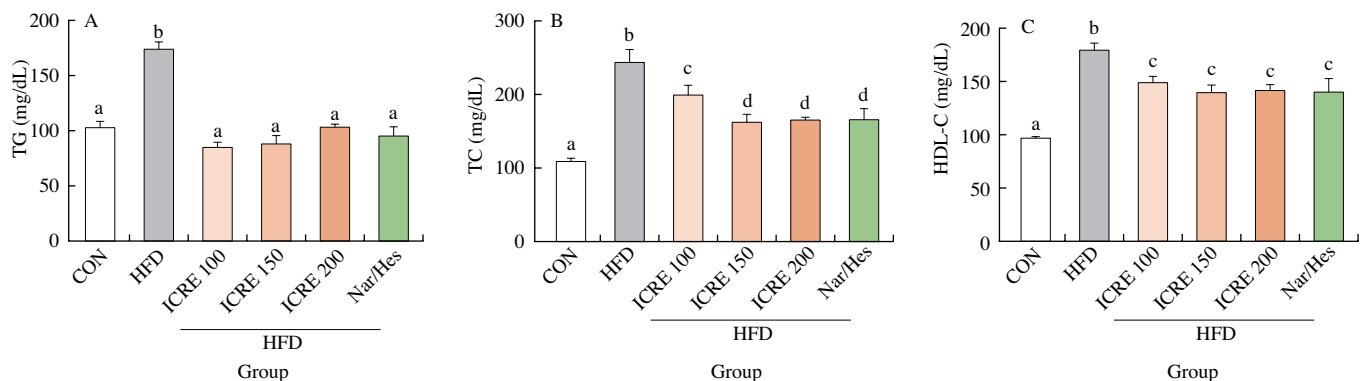


Fig. 3 Effects of ICRE on serum lipid profile and glucose levels. Serum levels of (A) TG, (B) TC, (C) HDL-C, and (D) LDL-C were measured in mice. (E) AUC of glucose during the oral glucose tolerance test. (F) Blood glucose levels were measured at 0, 15, 30, 60, and 120 min following glucose administration. (G, H) Serum levels of ALT, AST were measured to assess liver function. AUC, area under the curve.

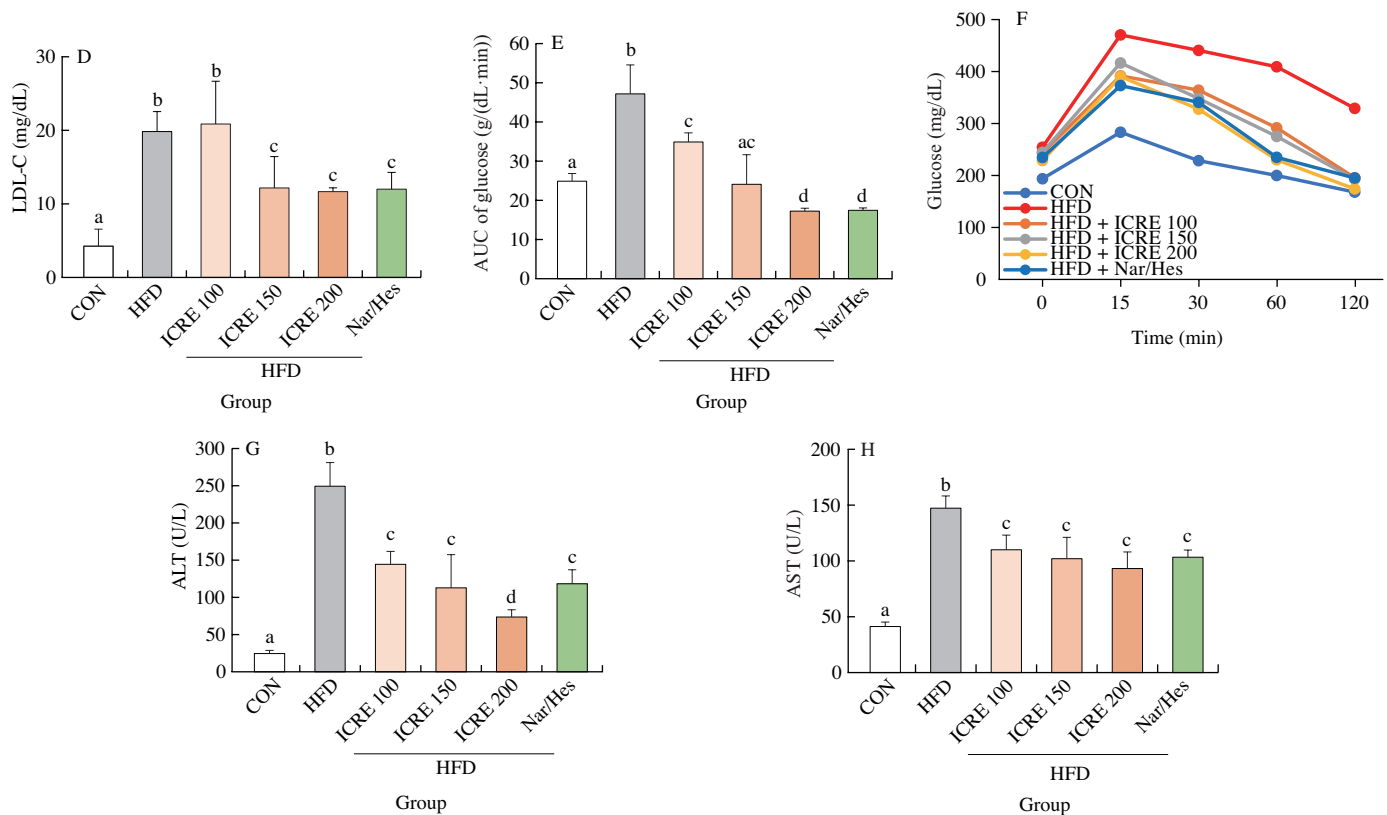


Fig. 3 (Continued)

qRT-PCR results showed that leptin mRNA expression levels were significantly increased in adipose tissue from the HFD group compared with that from the control group, indicative of obesity-induced leptin resistance (Fig. 4A). Additionally, the expression of the leptin receptor was also significantly increased in the HFD group compared with the control group (Fig. 4B). Treatment with ICRE or Nar/Hes led to a significant reduction in both leptin and leptin receptor mRNA expression levels compared with those of the HFD group. These findings suggest that ICRE and Nar/Hes regulate energy metabolism by inhibiting fat accumulation. By lowering leptin levels, these agents may restore proper energy balance and

counteract the effects of an HFD. Furthermore, the HFD group demonstrated significantly elevated serum lipase activity compared with the control group, reflecting the increase in lipolytic activity associated with excess fat storage (Fig. 4C). Importantly, treatment with ICRE resulted in a dose-dependent decrease in lipase activity compared with that of the HFD group that was significant at higher doses. This indicates that ICRE, like Nar/Hes, may act as a natural lipase inhibitor. The more pronounced reduction in lipase activity induced by ICRE compared with Nar/Hes suggests a greater impact on lipid metabolism.

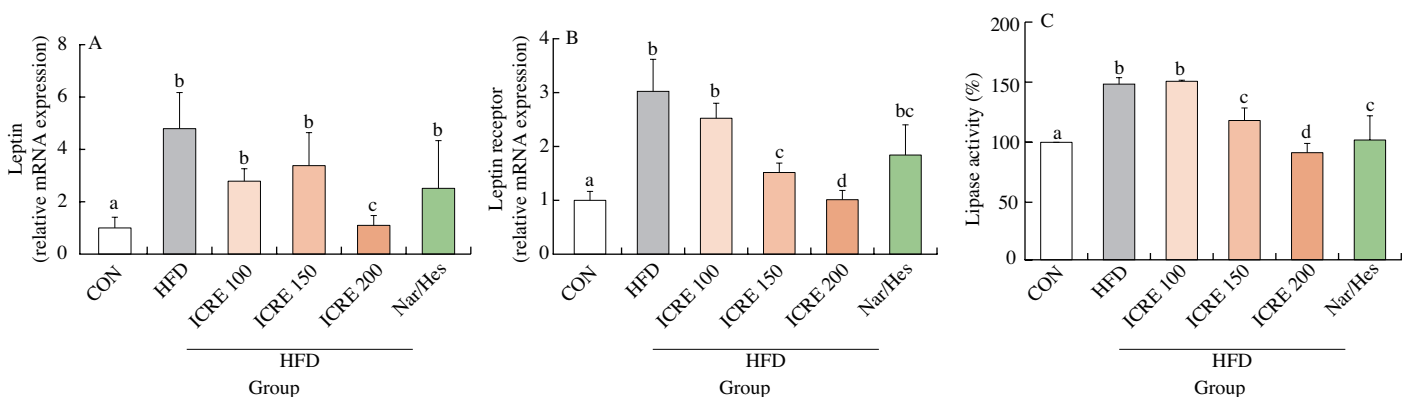


Fig. 4 Effects of ICRE on leptin levels and lipase activity. mRNA expression levels of (A) leptin and (B) leptin receptor in adipose tissue. (C) Serum lipase activity.

3.5 ICRE restores lipid metabolism and enhances energy expenditure in HFD-fed mice

To further clarify the effects of ICRE, we analyzed the expression of key genes involved in lipid metabolism. Genes were selected for their essential roles in lipid metabolism, adipogenesis, and energy regulation, allowing a comprehensive assessment of the anti-obesity effects of ICRE. qRT-PCR revealed significant alterations in the

expression of these regulators in adipose tissue in response to the HFD and treatment with ICRE or Nar/Hes (Fig. 5). Specifically, the HFD group exhibited elevated *SREBP-1*, *CEBP*, *PPAR γ* , *FAS*, *ACC*, *ACL*, and stearoyl-CoA desaturase 1 (*SCD1*) mRNA expression levels, indicating increased lipogenesis and impaired lipid homeostasis^[40]. Treatment with ICRE or Nar/Hes significantly inhibited these increases, suggesting modulation of adipogenic pathways. The 100 mg/kg ICRE group showed similar results to Nar/Hes, highlighting its

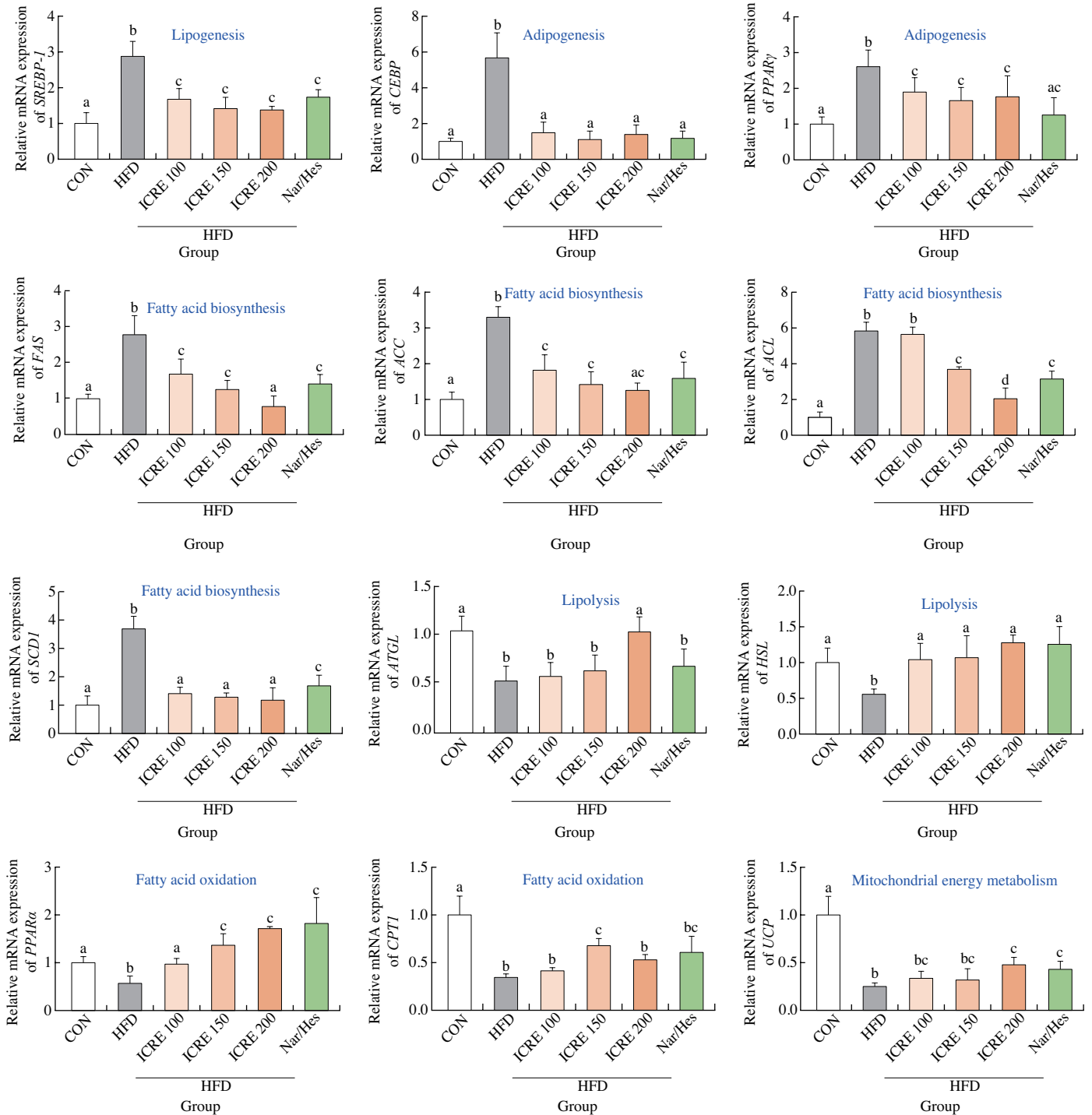


Fig. 5 mRNA expression levels of lipid metabolism-related genes in mouse adipose tissue. qRT-PCR was performed on adipose tissue to assess the mRNA expression levels of key genes involved in lipid metabolism.

efficacy at a lower dose. Conversely, the mRNA expression levels of adipose triglyceride lipase (*ATGL*) and hormone-sensitive lipase (*HSL*), which are crucial for lipolysis, were decreased in the HFD group compared with the control group; this decrease was inhibited by treatment with ICRE or Nar/Hes, indicating enhanced fat breakdown. Additionally, *PPARα* and carnitine palmitoyltransferase 1 (*CPT1*) mRNA expression levels were also fully or partially restored by treatment with ICRE or Nar/Hes, while uncoupling protein (*UCP*) levels were elevated by only a high concentration of ICRE, further supporting the potential of ICRE and Nar/Hes to promote metabolic health by improving lipid metabolism and energy expenditure.

3.6 ICRE promotes lipid metabolism regulation through AMPK activation in HFD-fed mice

To investigate the mechanism by which ICRE mediates its anti-obesity effects, we analyzed the expression levels of key proteins involved in lipid metabolism. Western blotting revealed that p-AMPK and total AMPK levels were significantly reduced in the HFD group compared with the control group, indicating impaired energy homeostasis (Figs. 6A and B). Treatment with ICRE or Nar/Hes resulted in a marked increase in both p-AMPK and total

AMPK levels compared with the HFD group. This increase suggests that ICRE and Nar/Hes may activate AMPK, which plays a pivotal role in regulating lipid metabolism and energy expenditure. The levels of p-AMPK were higher in the 200 mg/kg ICRE group than in the Nar/Hes group. In addition, the HFD group exhibited elevated levels of *PPARγ*, *SREBP-1*, and *FAS*, which are associated with lipogenesis and adipogenesis, further confirming obesity-induced metabolic dysregulation. Treatment with ICRE or Nar/Hes significantly reduced the expression levels of these proteins, suggesting that these agents can counteract the lipogenic and adipogenic effects of an HFD by promoting AMPK activation and inhibiting key pathways regulating fat synthesis. Both p-ACL and p-ACC levels were significantly elevated by ICRE or Nar/Hes treatment, indicating enhanced AMPK activity and suppression of lipogenic processes. The 100 mg/kg ICRE dose significantly altered *PPARγ*, *SREBP-1*, *FAS*, p-ACL, and p-ACC levels, indicating the effectiveness of ICRE even at a lower dose. These findings align with the established mechanism in which AMPK activation promotes fatty acid oxidation and inhibits lipogenesis, leading to reduced fat accumulation. Collectively, the results suggest that ICRE exerts its anti-obesity effects through AMPK activation and adipogenic regulator modulation, making it a potent modulator of lipid metabolism.

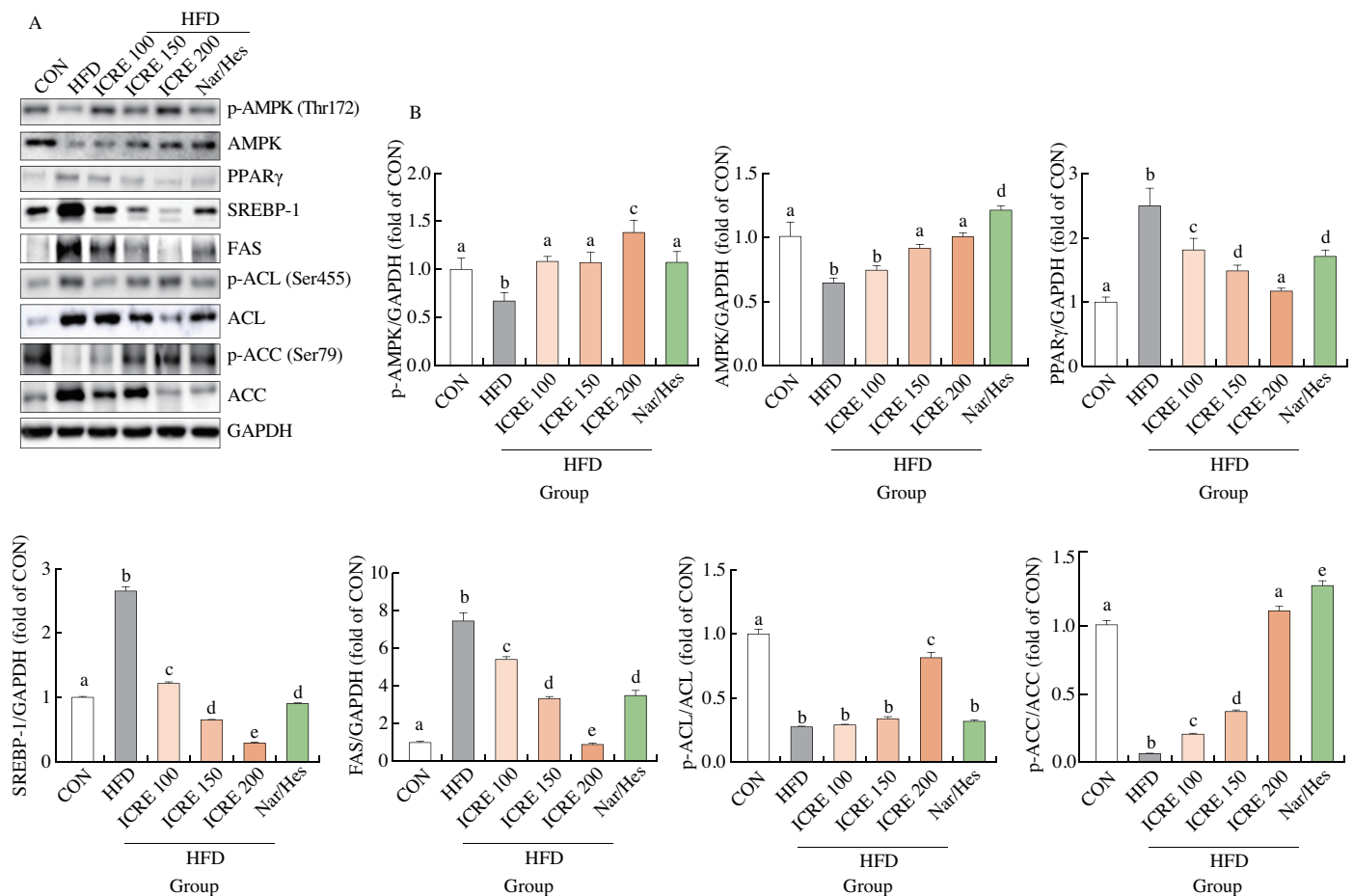


Fig. 6. Expression of proteins related to energy metabolism in mouse adipose tissue. (A) Western blots of key proteins involved in energy metabolism. (B) Quantification of protein expression.

4. Discussion

Several studies have revealed that ICRE offers distinct advantages over mature *C. reticulata* extract, primarily due to its higher concentrations of flavonoids, including hesperidin, narirutin, and nobiletin^[26-27,41]. These bioactive compounds contribute to superior antioxidant activity by scavenging free radicals, potentially preventing oxidative stress-related diseases^[42-43]. Additionally, ICRE has shown greater efficacy in inhibiting adipogenesis, suggesting enhanced potential for obesity prevention and management compared with mature citrus extract. This study aimed to investigate the anti-obesity potential of ICRE as well as a mixture of its key bioactive components, narirutin and hesperidin, in an HFD-induced obesity model^[12,15,44-45]. Our results demonstrated that ICRE exhibited significantly greater effects in reducing body weight, adipocyte size, and improving lipid metabolism markers than the Nar/Hes mixture. ICRE treatment resulted in a significant reduction in adipocyte size compared to the HFD group, with the 200 mg/kg ICRE group showing the most pronounced effect. Notably, the 200 mg/kg ICRE group exhibited a significantly greater reduction in adipocyte size than the Nar/Hes group. These findings suggest that the whole extract of ICRE contains synergistic compounds that amplify the metabolic benefits of narirutin and hesperidin, providing greater overall efficacy in combating obesity^[46-47]. This supports the hypothesis that whole plant extracts may provide greater therapeutic potential than isolated compounds by leveraging synergistic interactions among various phytochemicals.

ICRE not only regulates lipid metabolism but also improves glucose homeostasis in HFD-fed mice. The OGTT results indicate that ICRE effectively enhances glucose tolerance, demonstrating its potential to mitigate diet-induced impairments in glucose metabolism. The dose-dependent reduction in glucose levels suggests that ICRE improves systemic glucose regulation, potentially through enhanced insulin sensitivity. Given that AMPK activation plays a crucial role in glucose homeostasis, the observed effects may be attributed to ICRE-mediated AMPK activation, which enhances glucose uptake and utilization.

The key finding of this study is the effect of ICRE on the AMPK pathway, which plays a central role in energy homeostasis^[18,48]. AMPK activation by ICRE was associated with enhanced fatty acid oxidation, reduced adipocyte differentiation, and suppressed lipid synthesis. These results are consistent with those of previous studies, which showed that AMPK activation plays a crucial role in energy homeostasis by promoting catabolic processes while inhibiting lipogenic pathways^[19,49-52]. Our findings demonstrate that HFD consumption significantly increased *PPAR γ* and *SREBP-1* mRNA and protein levels in adipose tissue, while ICRE supplementation markedly inhibited this increase. This suggests that ICRE regulates lipid metabolism by activating AMPK, which in turn downregulates key transcription factors involved in lipid synthesis and adipogenesis. Based on our data, ICRE likely exerts its effects *via* AMPK activation, as evidenced by increased p-AMPK protein levels in adipose tissue, which coincided with the downregulation of *PPAR γ* and *SREBP-1*. AMPK has been well-documented to inhibit *SREBP-1* activity, thereby suppressing lipogenesis, while also modulating *PPAR γ* activity to regulate adipogenesis and lipid metabolism. These findings suggest that ICRE regulates lipid metabolism primarily through

AMPK rather than direct receptor interactions or post-translational modifications. While other pathways such as mammalian target of rapamycin signaling may contribute to lipid metabolism, our data indicate that AMPK activation is the primary mechanism underlying the metabolic effects of ICRE. Future studies will further elucidate whether additional signaling cascades also play a role. The phosphorylation of ACC and the reduced expressions of *SREBP-1* and *FAS* further support the role of ICRE in reducing lipid accumulation. These findings align with studies demonstrating that AMPK activation counteracts diet-induced obesity by promoting catabolic pathways and inhibiting lipogenesis^[32,53].

Furthermore, ICRE significantly increased AMPK activation while reducing leptin and leptin receptor expression levels in adipose tissue. Tukey's HSD test confirmed that the reduction in leptin levels was dose-dependent, with 200 mg/kg ICRE exhibiting the most significant effect compared to lower ICRE doses and Nar/Hes. Elevated leptin levels, commonly observed in obesity, contribute to leptin resistance, impairing energy homeostasis and promoting further weight gain^[38,54]. By decreasing the expressions of both leptin and its receptor, ICRE may help restore leptin sensitivity, thereby improving energy balance and supporting weight loss. Restoring proper leptin signaling is crucial for mitigating leptin resistance, a hallmark of metabolic dysfunction in obesity^[38,55]. Additionally, ICRE treatment led to a dose-dependent reduction in serum lipase activity, with a significant difference observed between the 200 mg/kg ICRE and Nar/Hes groups.

In addition to its effects on leptin signaling, the ability of ICRE to regulate lipase activity further supports its role in enhancing fat use and reducing fat storage. Lipase plays a key role in breaking down stored TG into free fatty acids, making them available for energy production. Excessive serum lipase activity was observed in the HFD group, potentially leading to increased lipid mobilization and metabolic stress^[18,56]. ICRE treatment led to a dose-dependent reduction in serum lipase activity, with a significant difference observed between the 200 mg/kg ICRE and Nar/Hes groups. ICRE supplementation significantly reduced serum lipase activity while increasing adipose tissue ATGL expression and p-AMPK activation. By modulating lipase activity, ICRE not only reduces lipid accumulation but also regulates lipid release, mobilization, and oxidation, providing a dual mechanism for its anti-obesity effects. This comprehensive metabolic regulation highlights the potential of ICRE as a natural therapeutic agent for obesity management, acting through multiple pathways to restore energy homeostasis and improve metabolic health.

The regulation of lipid metabolism is critical in obesity management, as dysregulation leads to excessive fat accumulation and metabolic dysfunction^[2-3,57-58]. Our findings demonstrate that ICRE effectively counteracts these metabolic disruptions by activating AMPK and modulating key regulators of lipid metabolism, including adipogenic, lipolytic, and energy expenditure pathways^[51-52]. HFD group mice exhibited increased expression of *SREBP-1*, *CEBP*, *PPAR γ* , *FAS*, *ACC*, *ACL*, and *SCD1*, key transcription factors and enzymes involved in lipogenesis. This upregulation promotes adipogenesis and lipid accumulation, contributing to obesity-related metabolic dysfunction. However, ICRE supplementation effectively suppressed these changes, indicating that ICRE counteracts excessive fat accumulation through transcriptional regulation of adipogenic

pathways. Furthermore, HFD-induced suppression of *ATGL* and *HSL*, key lipolytic enzymes, was reversed by ICRE treatment, suggesting enhanced lipid mobilization and utilization. AMPK activation is a key metabolic switch that enhances fatty acid oxidation while suppressing lipogenesis^[18,49–50]. HFD consumption led to a significant reduction in p-AMPK and total AMPK expression, reflecting impaired energy homeostasis, whereas ICRE treatment restored AMPK activation in a dose-dependent manner. Notably, the 200 mg/kg ICRE group exhibited the highest p-AMPK levels, surpassing those of the Nar/Hes group, indicating its stronger metabolic regulatory effects. Concurrently, ICRE significantly reduced the expression of PPAR γ , SREBP-1, and FAS, further confirming its role in suppressing adipogenic signaling cascades. Additionally, ICRE treatment led to significant increases in p-ACL and p-ACC levels, indicative of enhanced AMPK activity and inhibition of de novo lipogenesis. ACL and ACC are key enzymes in fatty acid and cholesterol biosynthesis, and their phosphorylation-mediated inhibition prevents excess lipid accumulation. Furthermore, only high-dose ICRE (200 mg/kg) significantly increased UCP expression, suggesting its role in promoting thermogenesis and energy dissipation. The observed effects were comparable or superior to those of Nar/Hes, suggesting that ICRE exerts a multi-targeted regulatory effect, likely due to its diverse bioactive components.

The efficacy of ICRE can be primarily attributed to key bioactive compounds such as narirutin and hesperidin^[8,59–60]. The antioxidant properties of hesperidin significantly reduce oxidative stress, while the anti-inflammatory effects of narirutin mitigate chronic inflammation often associated with obesity^[44,61–62]. These mechanisms are crucial, as oxidative stress and inflammation are major contributors to metabolic dysregulation and insulin resistance in obesity. Additionally, hesperidin enhances glucose uptake and improves insulin sensitivity in adipocytes^[63], thereby promoting metabolic health. The enhanced metabolic benefits of ICRE likely arise from synergistic interactions between various phytochemicals present in the extract^[64]. Especially, the balanced composition of narirutin and hesperidin in ICRE may contribute to its enhanced efficacy. Previous studies have identified hesperidin as the dominant compound in mature citrus extracts as we described in Section 3.1^[15]. However, our research revealed a more balanced concentration of narirutin and hesperidin ((12.6 ± 2.5) and (11.7 ± 2.3) mg/g, respectively). This balanced presence increases the likelihood of synergistic effects, enhancing the overall bioactivity of ICRE as a promising candidate for further research and development in anti-obesity treatments, marking a substantial advancement in the therapeutic use of citrus-based functional ingredients.

Plant-based extracts often exhibit enhanced efficacy because multiple components work on different metabolic pathways, an effect that is difficult to achieve with isolated compounds alone^[64]. This synergy may explain why ICRE shows greater anti-obesity potential than mature citrus extracts. Mechanistically, ICRE exerts broader and more potent metabolic regulatory effects compared to mature citrus extracts. It demonstrates superior activation of AMPK, a master regulator of energy homeostasis that stimulates fatty acid oxidation while inhibiting lipogenesis^[17–18,49]. This enhanced AMPK activation is likely due to the higher concentration and structural diversity of flavonoids in ICRE, particularly the glycosylated forms of hesperidin and narirutin, which are more abundant in immature citrus^[65–67]. In contrast, the ripening process

in mature citrus leads to the degradation or transformation of these bioactive compounds, thereby diminishing their efficacy in modulating metabolic pathways^[68–69]. Furthermore, ICRE more effectively suppresses lipogenic gene expression, including key regulators such as SREBP-1c and FAS, resulting in reduced lipid accumulation. ICRE also exerts stronger anti-inflammatory effects, likely due to its richer phytochemical profile, whereas mature citrus extracts with lower flavonoid content show relatively limited biological activity in these domains^[20,60]. Additionally, the presence of other bioactive compounds, some of which may remain unidentified, could further enhance the metabolic effects of narirutin and hesperidin. Future studies utilizing metabolomic profiling and fractionation analyses are needed to identify these key active components and clarify their contributions. Understanding these interactions could further reinforce the potential of ICRE as a therapeutic option for metabolic disorders.

This study has some limitations. First, although ICRE exhibited significant metabolic benefits, its long-term safety profile has not been fully established. Further studies are required to explore the chronic toxicity, pharmacokinetics, and bioavailability of ICRE to ensure its safety for prolonged consumption. Second, while we discussed potential mechanisms underlying the effects of ICRE, additional studies are needed to further elucidate its role in specific metabolic pathways, such as lipid metabolism, mitochondrial function, and gut microbiota interactions. Moreover, future studies should include clinical trials to assess the effectiveness of ICRE in humans and determine its potential as a functional ingredient in nutraceuticals or dietary supplements.

5. Conclusion

Given the global health burden of obesity and the limitations of current treatments, safe and effective alternatives are required. Natural products such as ICRE represent promising therapeutic options for obesity management owing to their multi-targeted effects on metabolic pathways and favorable safety profiles^[58,70]. This study demonstrates that ICRE exerts anti-obesity effects by activating AMPK, inhibiting lipogenesis, and promoting lipid oxidation, leading to reduced body weight gain, improved lipid profiles, and decreased adipocyte hypertrophy in mice with HFD-induced obesity. These findings highlight the potential of ICRE as a natural therapeutic agent for obesity management. Moreover, the ability of ICRE to target multiple metabolic pathways suggests its applicability as a functional ingredient in nutraceuticals or dietary supplements aimed at metabolic health improvement. While these results are promising, future studies are required to investigate the bioavailability, clinical efficacy, and long-term safety of ICRE in humans.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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