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Sea cucumber intestinal hydrolysates alleviate insulin resistance through regulating IRS1/PI3K/AKT signaling pathway mediated by glutamine metabolism in high-fat and high-sucrose diet-induced mice

Meng Wang[a], Man Yao[a], Hao Yue[b], Yingying Tian[a], Zifang Zhao[a,c], Changhu Xue[a,d], Tiantian Zhang[a,*], Yuming Wang[a,d,*]

[a] SKL of Marine Food Processing & Safety Control, College of Food Science and Engineering, Ocean University of China, Qingdao 266404, China
[b] Institute of Food & Nutrition Science and Technology, Shandong Academy of Agricultural Sciences, Jinan 250100, China
[c] Hainan Huayan Collagen Technology Co., Haikou 571000, China
[d] Sanya Institute of Oceanography, Ocean University of China, Sanya 572000, China

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ABSTRACT

It has been reported that sea cucumber intestine hydrolysates (SCIH) could promote glutamine metabolism in mice, while there is a close connection between glutamine metabolism and insulin sensitivity. However, the effect of SCIH on insulin resistance is still unclear. The results showed that SCIH hydrolyzed by flavor protease had significant activity using the insulin-induced HepG2 cell model. Animal experiments exhibited that SCIH supplementation significantly improved the high-fat and high-sucrose diet-induced impaired glucose tolerance, reduced fasting serum glucose and glycosylated serum protein. Besides, SCIH ameliorated islet vacuolization and decreased the pancreas TNF-α and IL-6 by 32.1% and 36.2%, respectively. Immunofluorescence staining results showed that SCIH promoted insulin secretion. Interestingly, SCIH significantly increased the liver glutamine levels and upregulated the expression of glutaminase1 (GLS1) and glutamate dehydrogenase 1 (GLUD1). Furthermore, SCIH increased liver acetyl-CoA levels to enhance histone acetylation and activate the gene transcription and translation on glucose metabolism-related IRS/PI3K/AKT signaling pathway, thereby attenuating insulin resistance. The present findings proposed the potential value for developing functional foods in SCIH utilization.

1. Introduction

Insulin resistance is clinically manifested as insufficient insulin secretion and reduced sensitivity of target organs to insulin, which is the pathological basis of obesity, diabetes and metabolic syndrome and other prevalent chronic metabolic disease[1-2]. Insulin receptor substrates (IRS), as the key target of insulin action, is usually activated by insulin to trigger a cascade reaction and exert its effects. Phosphatidylinositol 3-kinase (PI3K) is an important effector molecule of IRS, and activates AKT through phosphorylating serine/threonine-protein residues[3]. Subsequently, AKT inhibits glycogen synthesis kinase (GSK3β) through phosphorylation and leads to the activation of glycogen synthase (GS). In addition, AKT also participates in regulating the expression of gluconeogenic genes in liver tissues, especially forkhead box O1 (*FOXO1*), by

* Corresponding authors at: College of Food Science and Engineering, Ocean University of China, Qingdao 266404, China.
E-mail address: zhangtiantian@ouc.edu.cn (T.T. Zhang); wangyuming@ouc.edu.cn (Y.M. Wang)
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phosphorylating the FOXO transcription factor protein. Notably, the IRS/PI3K/AKT signaling pathway is inhibited during the insulin resistance stage. Therefore, activating the IRS/PI3K/AKT signaling pathway is an important target for improving insulin resistance. Recently, new evidence has found that histone acetylation is an important factor in insulin resistance, inflammatory response and diabetes[4]. As an acetylation protein, IRS-1 can be acetylated and modified by histones, which further promotes tyrosine phosphorylation and further insulin-stimulated signal transduction[5]. Moreover, animal experiments showed that glutamine supplementation significantly improved blood glucose levels. Interestingly, acetyl CoA, a glutamine metabolite, is also the key substrate for histone acetylation. Consequently, glutamine metabolism is an important target for the treatment of insulin resistance. Recently, dietary supplementation with bioactive peptides exhibited the functions on improving insulin resistance, anti-diabetes, and regulating glucose and lipid metabolism[6].

Sea cucumber is a medicinal and edible aquatic product with high nutritional value, which is highly praised for its rich amino acids, vitamins and various biologically active substances[7]. The sea cucumber intestines accounts for approximately 30%–40% of its overall weight and are rich in protein, accounting for 70% of dry weight[8]. Therefore, it is necessary to investigate and develop the nutritional functions on its proteins and peptides. In addition, enzymatic hydrolysis is the most widely used method for preparing active peptides, which has the advantages of simple operation, mile conditions and high safety. The commonly used enzymes include flavor proteases, alkaline proteases, neutral proteases, papain and so on. Considering the abundant protein content of sea cucumber intestines, the preparation of sea cucumber intestine hydrolysates (SCIH) is promising as a useful method to develop functional food ingredient. Yue et al.[9] found that SCIH promoted longitudinal bone growth through regulating the glutamine metabolism in adolescent mice. Moreover, sea cucumber peptides have been found to promote the acetylation level of histones 3 in the hippocampus, thereby enhancing the memory ability[10]. There is a close connection between glutamine metabolism and insulin sensitivity, however, it is still unclear that whether SCIH have a positive effect on alleviating insulin resistance.

Here, the sea cucumber intestines were firstly hydrolyzed into peptides by different proteases, and the optimal protease hydrolysates for alleviating insulin resistance were further screened by evaluating glucose consumption using an insulin-induced HepG2 cell model. Then, high-fat and high-sucrose diet (HFSD) fed C57BL/6J mice were used to explore the nutritional function of dietary SCIH on insulin resistance by investigating the oral glucose tolerance and glucose metabolism level in liver. Furthermore, the possible underlying mechanisms were elucidated.

2. Materials and methods

2.1 Preparation of SCIH

Sea cucumber (*Apostichopus japonicus*) intestines were obtained from Shandong Haodangjia Ocean Development Co., Ltd. (Weihai, China). The sea cucumber intestines were homogenized with deionized water at a solid-to-water ratio of 1∶5 (*V*/*V*). The homogenate was carried out with 1.8% alkaline protease (pH 9.0, 55 ℃) for 5.6 h, 1.5% neutral protease (pH 7.0, 55 ℃) for 7.2 h, and 1.6% flavor protease (pH 6.0, 55 ℃) for 6 h, respectively[11-12]. The used commercial proteases were purchased from Solarbio (Shanghai, China). Then, the hydrolysis process was stopped by boiling for 15 min. After cooling, the pH of all hydrolysates was adjusted to neutral. Afterwards, the supernatant was further fractioned by ultrafiltration with a molecular weight membrane of 3 kDa to collect the filtrate, which was desalinated and freeze-dried to light yellow powder. All other chemicals and reagents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). The alkaline hydrolysates were SCIH-1, the neutral hydrolysates were SCIH-2, and the flavor protease hydrolysates were SCIH-3.

2.2 Amino acid composition analysis

The amino acid composition was measured following the previous method[9]. Briefly, all samples were subjected to deproteinization and proceed with 6 mol/L HCl at 110 ℃ for 22 h. Subsequently, the determination was performed by an automated amino acid analyzer (835-50, Hitachi).

2.3 Cell culture and treatment

The HepG2 cells (ATCC, Manassas, VA, USA) were cultured in minimum essential medium (MEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at a temperature of 37 ℃, under a controlled atmosphere of 5% CO_2 and 95% relative humidity. The insulin-resistant cell model was induced using modified methods previously described[13]. Briefly, the HepG2 cells after cell adhesion were treated with different concentrations of insulin (0.2, 1.0, 5.0, 25.0, 125.0, 625.0 μg/mL) (Sigma, St. Louis, MO, USA) for 48 h to investigate the effect of insulin on HepG2 cell viability. The appropriate concentration of insulin was selected by measuring the cell viability for modeling.

After cell adhesion, each experimental group was cultured with 1 mL of insulin at different concentrations (0, 0.2, 1.0, 5.0, 25.0 μg/mL) for 24 and 48 h, respectively, and the sugar concentration in each group of cells was 25 mmol/L. The cellular glucose consumption was assayed in the medium with a glucose assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the glucose utilization (from 24 to 48 h) were calculated as follow:

Glucose consumption (mmol/L) =

Glucose content in normal group – Glucose content in experimental group (1)

$$\text{Glucose utilization} = \frac{\text{48 h glucose consumption} - \text{24 h glucose consumption}}{\text{24 h glucose consumption}} \quad (2)$$

2.4 Cell viability assay

The cell viability was conducted according to the Methyl thiazolyl tetrazolium (MTT) (Sigma, St. Louis, MO, USA) method[14-15]. Briefly, HepG2 cells were plated in a 96-well plate at a density of 2.0×10^4 cells/mL and incubated for 12 h. The culture medium was removed, and 150 μL of SCIH-1, SCIH-2 and SCIH-3, respectively, at different concentrations (0.0, 12.5, 25.0, 50.0, 100.0, 200.0, 400.0 μg/mL) were added to each well, with 6 replicates per group and cultured for

24 h. The HepG2 cells were treated with MTT solution (100 μL, 0.5 mg/mL) for 4 h. Next, the MTT solution was replaced by DMSO, and the plates were shaken for 10 min to dissolve all blue-violet crystals, which were measured at a wavelength of 490 nm to estimate the cell viability. Then the effects of insulin were tested.

2.5 Effect of SCIH on cellular glucose consumption

The HepG2 cells were divided into 5 groups with or without insulin in medium for further research: control group (without insulin), insulin (INS) group, and hydrolysates group induced by insulin together with different concentrations of SCIH-1, SCIH-2 and SCIH-3 (0, 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL, dissolved in complete medium). The cells seeded into a 96-well plate (2.0×10^4 cells/mL) for 24 h. Then the glucose concentrations in the supernatants were measured with kit to further determine the optimal sea cucumber intestine hydrolysates.

2.6 Animals experiment.

Male C57BL/6J mice (SPF, aged 7 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in specific cages at (22 ± 2) ℃ with a 12-h light/dark cycle. Animal experiments were conducted in accordance with the Guidelines for Animal Protection and received approval from the Experimental Animal Care Ethics Committee of Ocean University of China (Certificate number: SYXK20120014). According to the results of cell experiments, SCIH-3 (hydrolyzed by flavor protease) was used for further animal experiments. After 1 week of acclimatization, a mouse model of insulin resistance was established by feeding a HFSD for 14 weeks, in which sugar and fataccounted for 20% and 25%, respectively. Table S1 listed the composition of low-fat and low-sucrose diet (LFSD) and HFSD, respectively.

Mice were randomly divided into 4 groups (n = 8, per group) including: the normal group (Normal), the model group (Model), the Met group (metformin, 200 mg/kg BW), and the SCIH supplementation group (SCIH, 500 mg/kg BW). A LFSD were given to mice in the normal group, while other groups were given HFSD. During the dietary induction, the mice were administered orally with 1 mL/100 g BW once a day for 100 days and the body weight was recorded. The mice were fasted overnight for 8 h, and then sacrificed under isoflurane anesthesia to collect blood by eyeball extraction for serum isolation. Mice serum concentrations of insulin and glycosylated serum protein (GSP) were measured by ELISA kits (Millipore, Bedford, MA, USA), respectively. The liver and pancreas tissues were immediately dissected and stored at –80 ℃.

2.7 Oral glucose tolerance test (OGTT)

An OGTT was conducted on the week before mice sacrificed as described previously[16]. After mice were administered 0.2 g/mL glucose, serum samples were obtained from the tail vein at 0, 0.5, 1.0, and 2.0 h using the glucose assay kit (Biosino Co., Ltd., Beijing, China). The serum glucose value at 0 h represented the fasting serum glucose level during OGTT.

2.8 Analysis of homeostatic model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment for beta-cell function (HOMA-β)

Based on the levels of serum indexes above, the HOMA-IR and HOMA-β were calculated by formula in accordance with Li et al.[17]:

$$\text{HOMA-IR} = \frac{\text{Fasting insulin (μU/mL)} \times \text{Fasting glucose (mmol/L)}}{22.5} \quad (3)$$

$$\text{HOMA-β} = \frac{20 \times \text{Fasting insulin (mIU/L)}}{\text{Fasting glucose (mmol/L)} - 3.5} \quad (4)$$

2.9 Measurement of pancreas inflammation levels

According to Liu et al.[18], pancreas tissue (about 0.1 g) was ground into powder by liquid nitrogen, and the tissue was homogenized according to 1:9 in an enzyme-free EP tube with PBS, and further centrifuged at 5 000 r/min. The supernatant was used to determine the total protein concentration by BCA kit (Solarbio, Beijing, China). The tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels were examined using mouse ELISA kits (Millipore, Bedford, MA, USA).

2.10 Biochemical analysis in liver

The liver tissue (0.1 g) was broken into lye homogenate, and the glucose assay kit. The liver glutamine and acetyl-CoA levels were measured by ELISA kits (Millipore, Bedford, MA, USA). The operations followed the instructions of the manufacturer.

2.11 Histopathological examination.

Pancreas tissues were fixed in 10% neutral formaldehyde overnight, followed by dehydration with graded ethanol, clearing with xylene, and embedding in paraffin. The samples were sectioned and subsequently stained with hematoxylin and eosin (H&E). In addition, the liver tissue was also stained with periodic acid-Schiff (PAS). The morphological and pathological changes of liver and pancreatic tissue sections were observed under the microscope (Olympus, Japan).

2.12 Immunofluorescence staining

Paraffin-embedded slices were dewaxed and rehydrated, immersed in EDTA buffer (pH 8.0), and heated in a microwave to enhance antigen retrieval. Then, the sections were sealed with bovine serum albumin and sequentially incubated with primary antibodies (insulin and glucagon), as well as the secondary antibodies, followed by staining with nucleic acid dye DAPI. The nuclei were dyed with blue fluorescence signal, while insulin was labeled with red fluorescence by CY3, and the glucagon exhibited green fluorescence by fluorescein isothiocyanate (FITC). The ImageJ software was used for semi-quantitative analysis of insulin-Met cells in each section.

2.13 Determination of mRNA expression levels by qRT-PCR

The related mRNA expression in liver were performed by qRT-PCR. Briefly, total mRNA from frozen liver tissue was isolated respectively in each group using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed to cDNA. The genes were evaluated

using SYBR Green Master (Toyobo, Osaka, Japan) in 25 μL of reaction system. The expression of *β*-actin was applied as a normalization reference. The primers were list in Table S2, respectively, which were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

2.14 Western blot analysis

The frozen liver tissues (300 mg) were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA) and centrifuged to obtain total protein. The protein concentration was assayed by the BCA protein assay kit (Biosino, Beijing, China) before electrically transferred onto a PVDF membrane through a 10% SDS-PAGE gel. Further, these membranes were blocked with 5% bovine serum albumin, and incubated with the primary antibodies, including histone 3 (H3, 1:2 000), histone 3K9 acetylation (H3K9Ac, 1:500), IRS-1 (1:1 500), IR, p-IRS-1, PI3K, AKT, p-PI3K, p-AKT, GSK3β, p-GSK3β, FOXO1, p-FOXO1-ser256, PGC-1α, PEPCK, glutaminase 1 (GLS1) (1:1 000), glutamate dehydrogenase 1 (GLUD1, 1:5 000), and *β*-actin (1:4 000). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). After washing with TBST, the membranes were incubated with secondary antibodies conjugated to HRP. The ECL stain kit (Amer sham Biosciences Corp., Piscataway, NJ, USA) was utilized to visualize the final results of the protein bands.

2.15 Statistical analysis

All experiments were biologically repeated for triplicate and presented as the mean ± standard error of the mean (SEM). The statistical analysis of multiple comparisons among each group was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. A value of $P < 0.05$ was defined as statistically significant, and $P < 0.01$ as extremely significant difference. GraphPad prism 8.0 was used to all analyses.

3. Results

3.1 Insulin resistance model (IRM) development

To obtain the IRM of HepG2 cells, we investigated the effect of different concentrations of insulin on cell viability and glucose concentration. As shown in Fig. S1A, the cell viability of HepG2 decreased when insulin concentration was 25–625 μg/mL, while it was increased in 0.2–25.0 μg/mL, indicating that insulin concentration of 0.2–25.0 μg/mL had a non-toxic effect. As shown in Fig. S1B, the cellular glucose consumption slowly increased when incubating for 24 h, while cells incubated with 5 μg/mL insulin for 48 h showed the lowest cellular glucose consumption when compared to the cells without insulin treatment. Meanwhile the glucose utilization (Fig. S1C) exhibited a gradual decrease with increasing insulin concentration and the utilization rate was the lowest at 5 μg/mL insulin. Hence, treatment of HepG2 cells with 5 μg/mL insulin for 48 h was finalized to conduct HepG2/IRM cells.

3.2 SCIH enhanced glucose consumption in HepG2 cells

The MTT results showed that the cell viability of HepG2 cells were not affected by 3 different concentrations of SCIH hydrolysates, indicating non-toxicity and suitability for further investigation (Fig. 1A). As shown in Fig. 1B, when compared to Control group, the glucose consumption in INS group cells was significantly decreased ($P < 0.01$). SCIH-1 (200 μg/mL) and SCIH-3 (100 and 200 μg/mL) remarkably promoted glucose consumption, among which the SCIH-1was 21.6% and the SCIH-3 was 26.9% and 44.8%, respectively, while no significant difference was observed in SCIH-2 group, indicating that both SCIH-1 and SCIH-3 exhibited significant effects in alleviating insulin resistance. Interestingly, the effect of SCIH-3 at 100 μg/mL glucose consumption was similar to SCIH-1 at a concentration of 200 μg/mL in HepG2 cells. To sum up, the SCIH-3 hydrolyzed by flavor protease was selected for subsequent experiments.

3.3 Amino acid composition of SCIH

The functional activity of small molecule hydrolysates is determined by their specific amino acid composition and sequence. Based on the above, we determined the amino acid composition of SCIH-3 (hereinafter collectively referred to as SCIH). The results revealed that the molecular weight of SCIH was mainly below 1 000 Da (88.9%), above which the majority were short peptides with a molecular weight less than 500 Da (Fig. 2A). The biological activity of peptides was determined by

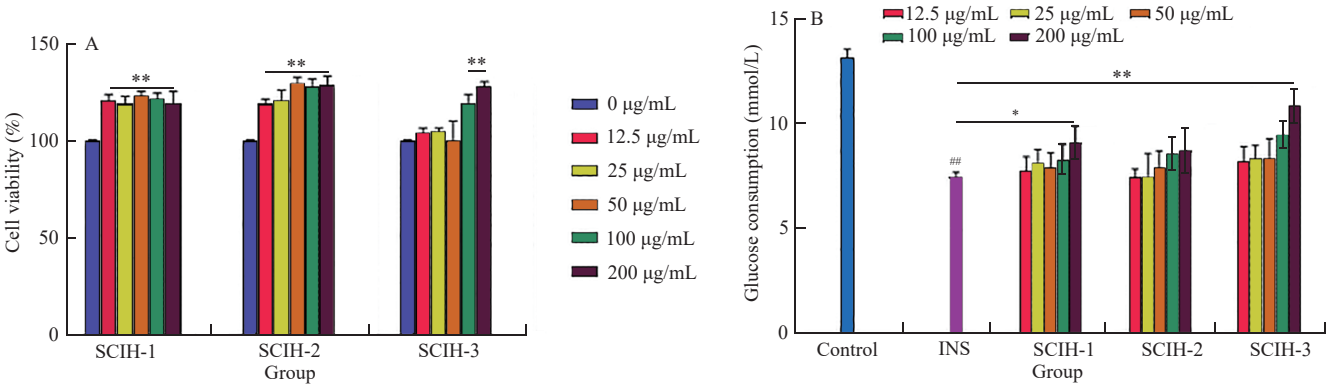

Fig. 1 Establishment of the IR-HepG2 model induced by high-insulin. (A) Effect of SCIH on the cell viability of HepG2 cells. (B) Effect of SCIH on glucose consumption in HepG2 cells induced by insulin. Data were expressed as mean ± SEM. Statistical significance $^{\#\#}P < 0.01$ compared with the normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ among all groups determined by ANOVA.

their differences of amnio acid composition and conformation. Total of 17 amino acid constituents were detected in the SCIH sample, and the content of the essential amino acids accounted for 34%. Importantly, glutamate (16%), aspartic acid (12%), glycine (9%) arginine (8%) and leucine (8%) were the major amino acids (Fig. 2B).

3.4 SCIH improved oral glucose tolerance and decreased serum glucose

Weight gain and fasting serum glucose level in mice fed a HFSD are 2 important factors for insulin resistance. Insulin is an important endogenous hormone for maintaining serum glucose homeostasis. Compared to the Normal group, the mice weight gain was dramatically increased ($P < 0.01$) in Model group, while significantly decreased both in Met group and SCIH group (Fig. 3A). The fasting serum glucose and the fasting serum insulin level were significantly increased by 56.4% and 39.4% ($P < 0.01$) in the Model group, compared with Normal group (Figs. 3B and C). Obviously, SCIH and metformin treatment could decrease the serum insulin level by 16.2% and 19.6%, respectively. However, the serum insulin level both in the Met group and the SCIH group was observed no significant difference. GSP level reflects the average serum glucose level in the past 2 weeks, which is independent of temporary fluctuations in serum glucose concentration. The GSP levels in the Model group were increased by 63.5% ($P < 0.01$), while it was reduced by 16.4% in the SCIH group (Fig. 3D).

The OGTT was preformed to reflect short-term changes in serum glucose level and the effect of SCIH on insulin resistance. As shown in Fig. 2E, the serum glucose level peaked at 0.5 h after taking administration of glucose orally and markedly decreased at 2 h. In addition, mice in Model group exhibited higher serum glucose at 0.5 h when compared to the Normal group, and the glucose AUC increased by 33.7%, indicating that HFSD impaired glucose tolerance evidently (Fig. 3F). SCIH markedly decreased serum glucose level at 0.5 h, and the glucose AUC reduced by 11.1% ($P < 0.05$). These data suggested that SCIH had an effective function on lowering serum glucose in HFSD-induced mice.

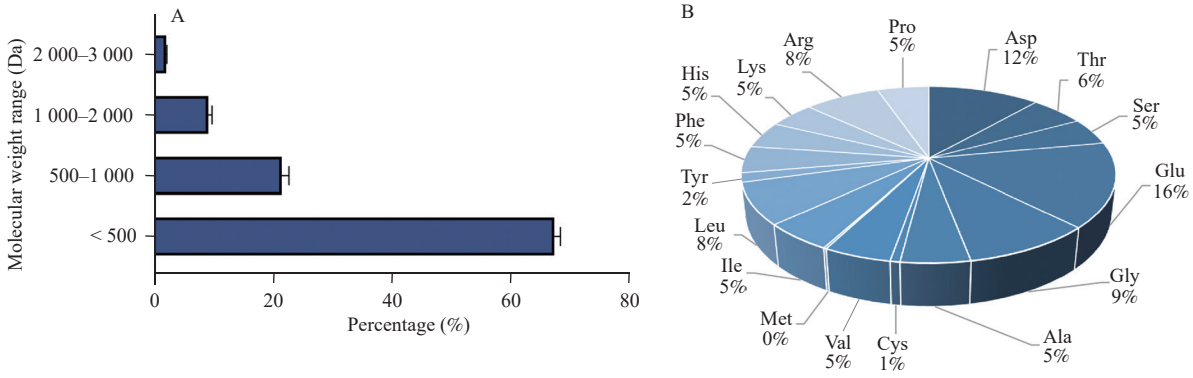

Fig. 2 Composition analysis of SCIH. (A) Molecular weight distribution of SCIH; (B) amino acid composition of SCIH. Data were expressed as mean ± SEM.

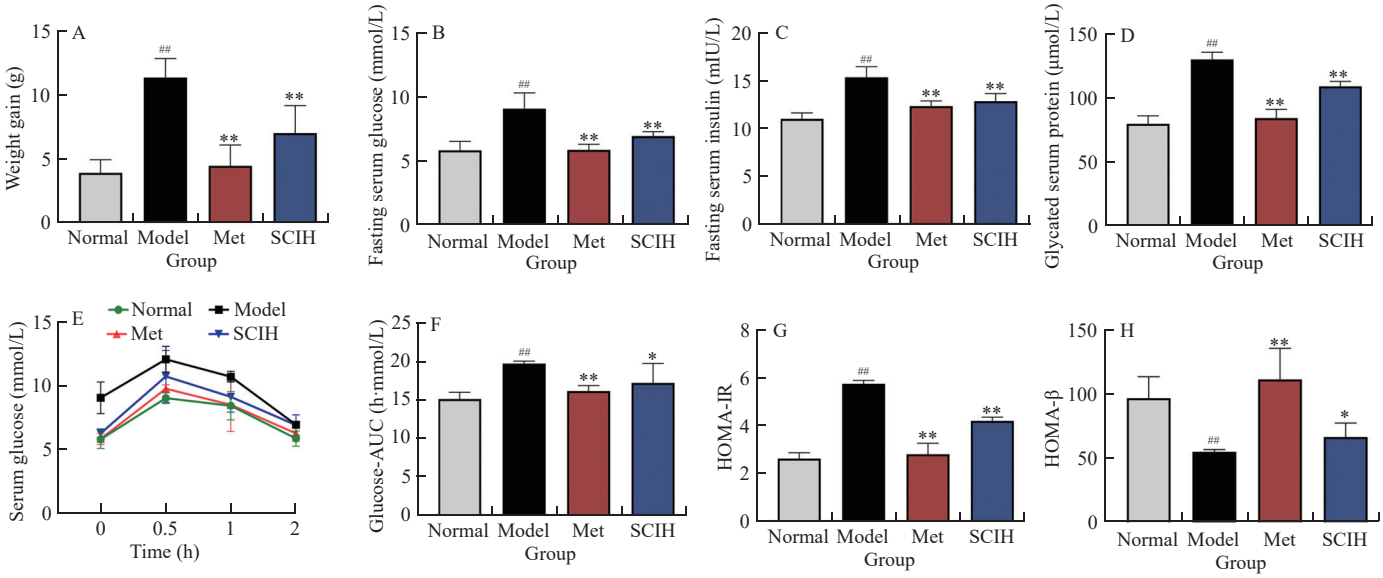

Fig. 3 Effects of SCIH on alleviating insulin resistance in HFSD-fed mice. (A) Body weight gain. (B) Fasting serum glucose level. (C) Fasting serum insulin level. (D) GSP level. (E) Serum glucose. (F) AUC of serum glucose. (G) HOMA-IR index. (H) HOMA-β index. Data were expressed as mean ± SEM ($n = 8$). $^{\#\#}P < 0.01$ compared with the Normal group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the Model group.

3.5 SCIH increased serum insulin levels and improved HOMA-IR and HOMA-β

The HOMA-IR and HOMA-β are crucial for reflecting insulin resistance itself and insulin secretion of pancreas, respectively. According to the data, dietary SCIH reduced the HOMA-IR index by 26.7%, and increased HOMA-β index by 21.2% ($P < 0.01$) when compared to the Model group (Figs. 3G and H). Therefore, the above results implied that supplementation with SCIH effectively improved pancreatic islet β cell dysfunction.

3.6 SCIH alleviated pancreatic islet damage and enhanced insulin secretion

The damage of pancreatic microstructure directly leads to the disorder of islet β cells and promotes insulin resistance. In the Model group, the islets were atrophic along with the apoptotic cells and obvious vacuolization (Fig. 4A). In the Normal group and the SCIH group, islet cells were relatively regular with fewer cytoplasmic vacuoles. This phenomenon illustrated that SCIH had the ability to alleviate the microstructural damage of islets caused by insulin resistance. The fluorescence intensity of insulin in the Model group was lower than that in the normal group (Fig. 4B), but the fluorescence intensity of glucagon was just opposite. The insulin semi quantitative results revealed that the fluorescence intensity of insulin decreased by 39.7% significantly in the Model group, when compared with the Normal group. Notably, compared to the Model group, SCIH supplementation increased that by 28.6% ($P < 0.01$) (Figs. 4B and C). Typical cellar inflammatory factors mediate the inflammatory response through complex interactions to promote apoptosis and dysfunction of pancreatic β cells, ultimately affecting insulin secretion[17]. As shown in Figs. 4D and E, the TNF-α and IL-6 levels in pancreas tissue of the Model mice was significantly increased, confirming that HFS diet induced chronic inflammation of the pancreas. Interestingly, dietary SCIH reduced by 32.1% and 36.2%, respectively. The results suggested that dietary SCIH effectively enhanced islet function and stimulated insulin secretion in mice *via* ameliorating the inflammatory environment.

3.7 SCIH accelerated liver glycogen synthesis

Maintaining fructose metabolic homeostasis primarily occurs in the liver, and the interplay between hepatic glycogen synthesis and gluconeogenesis is essential for the development of metabolic syndrome[19]. Mice liver glycogen levels were examined by PAS glycogen staining (Fig. 5A). It was evidently viewed that the liver glycogen was highlighted by PAS staining in the normal group and the SCIH group, while few in the Model group. The results of glycogen assay kit confirmed again that the liver glycogen content was remarkably decreased by 51.9% ($P < 0.01$) in the Model group when compared to the Normal group. Notably, dietary SCIH could enhance the liver glycogen level by 51.6% (Fig. 5B). The enzymes glucokinase (GK) and hexokinase (HK) play a crucial role in regulating glycogen synthesis in liver by catalyzing the phosphorylation process of glucose[20]. Compared with the Model group, both GK and HK levels exhibited an increase expression in SCIH group, indicating that SCIH supplementation effectively enhanced the liver glycogen synthesis in HFSD-induced mice (Fig. 5C).

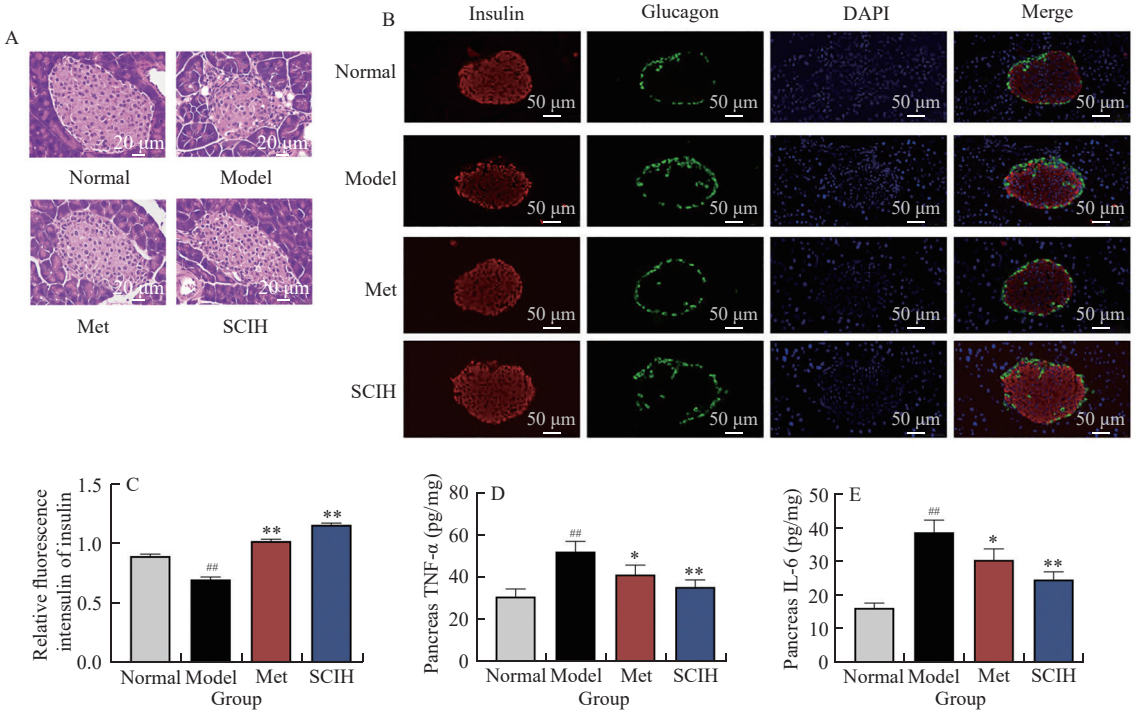

Fig. 4 Analysis of pancreatic H&E staining, insulin-glucagon immunofluorescence staining, and the inflammation levels of pancreas. (A) H&E staining, scale bar = 20 μm. (B) Pancreatic immunofluorescence staining showing the presence and distribution of insulin-producing (insulin, red) cells, glucagon-producing (glucagon, green) cells, and nucleus (DAPI, blue), scale bar = 50 μm. (C) Semiquantitative analysis of insulin positive cells. (D) Pancreas TNF-α. (E) Pancreas IL-6. Data were expressed as mean ± SEM ($n = 8$). $^{\#\#}P < 0.01$ compared with the Normal group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the Model group.

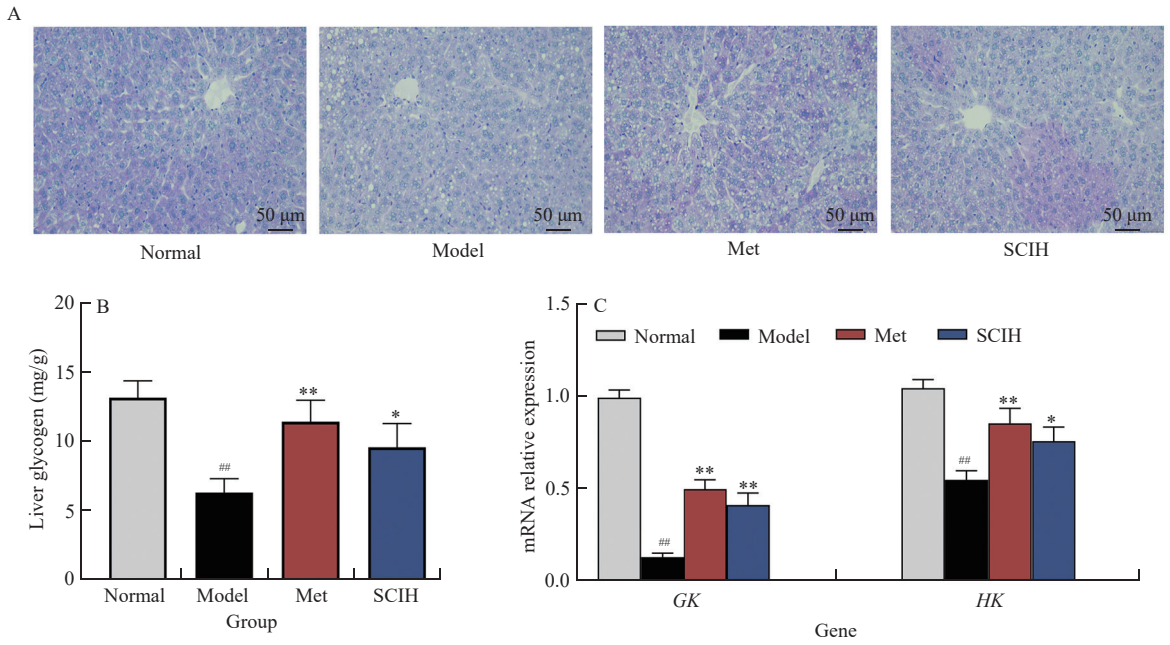

Fig. 5 Effect of SCIH on hepatic glycogen. (A) PAS staining of mice liver, scale bar = 50 μm. (B) Liver glycogen levels. (C) mRNA expression levels of key enzymes *GK* and *HK* for glycogen synthesis. Data were expressed as mean ± SEM (n = 8). $^{\#\#}P < 0.01$ compared with the Normal group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the Model group.

3.8 SCIH improved hepatic glucose metabolism

At the transcription level, the mRNA expression of *PI3K*, *AKT* and *GS* were significantly downregulated, and the *GSK-3β* was upregulated in the Model group. However, the genes expression was significantly reversed in the SCIH group (Fig. 6A). In terms of the protein level, the ratio of p-PI3K/PI3K in Model group was significantly reduced, while supplementation with SCIH increased the ratio expression ($P < 0.05$). Compared to the Normal group, the ratio of p-AKT/AKT in Model group was significant increased, however, there is no statistically significant difference between SCIH group and Model group ($P > 0.05$) (Figs. 6B and C).

The key genes and proteins were further examined on the FOXO1/PGC-1α signaling pathway in mice liver. The results found that HFSD activated genes expression on the FOXO1/PGC-1α signaling pathway, which significantly upregulated the expression of *FOXO1* and *PGC-1α* by 2.3- and 1.9-flod, respectively, and the downstream genes *G-6-Pase* and *PEPCK* (1.5- and 1.6-flod, respectively) compared to that in the Normal group (Fig. 6D). Interestingly, SCIH downregulated the mRNA expression of *FOXO1*, *PGC-1α*, *G-6-Pase* and *PEPCK* by 26.3%, 29.8%, 24.1% and 20.6%, respectively, compared to the Model group ($P < 0.01$). Besides, the results of Western blot revealed that SCIH supplementation efficiently suppressed the protein levels of FOXO1, PGC-1 and PEPCK, compared to the Normal group ($P < 0.01$) (Fig. 6F). The p-FOXO1 Ser256/FOXO1 ratio exhibited an increase in the SCIH group as compared with that in the Model group, which meant an upregulation in FOXO1 phosphorylation and its subsequent nucleus translocation process (Fig. 6E). Therefore, the protein level of the downstream key enzyme PEPCK for hepatic gluconeogenesis was also reduced following SCIH supplementation. The above results suggested that SCIH promoted hepatic glycogen synthesis and inhibited gluconeogenesis through the GSK-3β/PI3K/AKT and FOXO1/PGC-1α signaling pathway to alleviate insulin resistance.

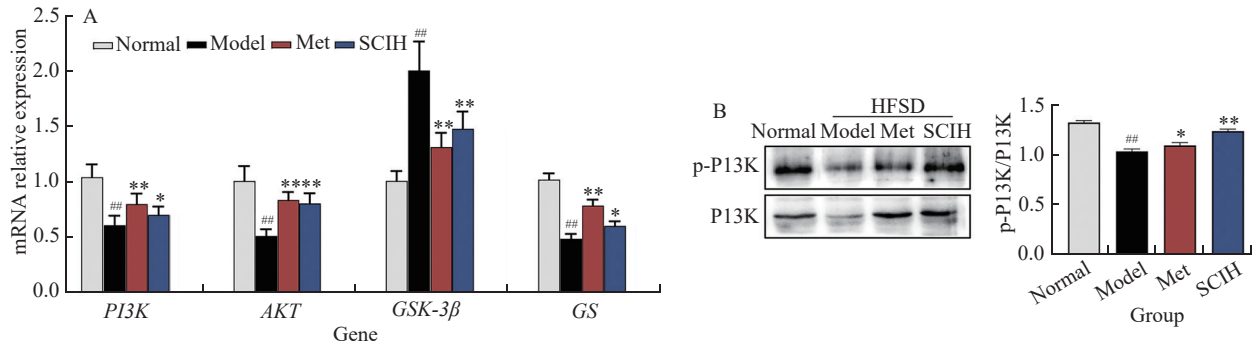

Fig. 6 mRNA and protein expression of the key genes on liver glucose metabolism in HFSD-fed mice. (A) mRNA expressions of *PI3K*, *AKT*, *GSK-3β* and *GS*. Protein expressions of (B) p-PI3K/PI3K and (C) p-AKT/AKT. (D) mRNA expressions of FOXO1/PGC-1α pathway. (E) p-FOXO1/FOXO1. (F) Protein expressions of PGC-1α, PEPCK and G-6-Pase. Data were expressed as mean ± SEM (n = 8). $^{\#\#}P < 0.01$ compared with the Normal group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the Model group.

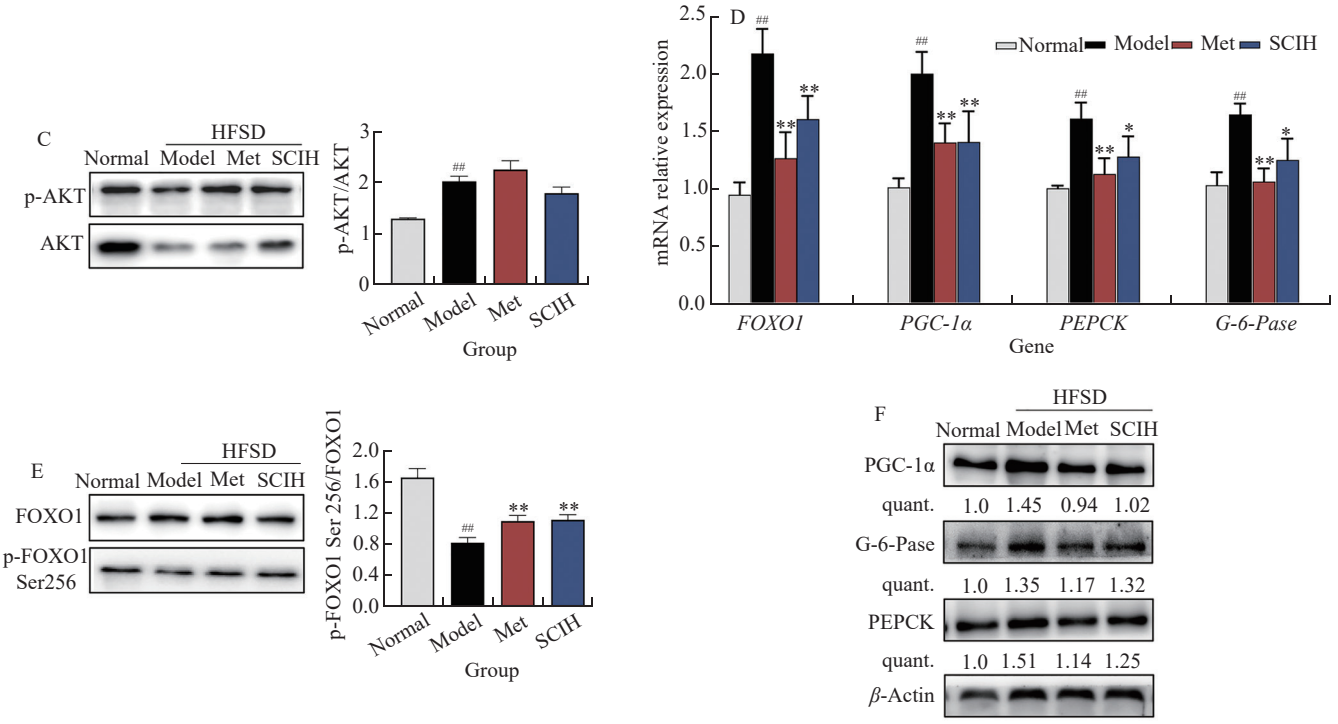

Fig. 6 (*Continued*)

3.9 SCIH promoted the transcription and translation of liver IRS1 by acetylated histone

The specific environment of liver enables glutamine metabolism to exhibit an enormous function for histone acetylation modification. Our previous study has proved that SCIH supplementation exerted a significant effect on glutamine metabolism[9], therefore, the glutamine levels were determined in liver. As shown in Fig. 7A, the liver glutamine level was significantly increased in the SCIH group in comparison with the Model group. Besides, supplementation with SCIH significantly increased the protein levels of key glutamine catabolizing enzymes GLUD1 and GLS1, respectively, which further resulted in a dramatical increase of acetyl-CoA (Fig. 7B and C), a glutamine metabolite. Acetyl-CoA is the primary substrate for histone

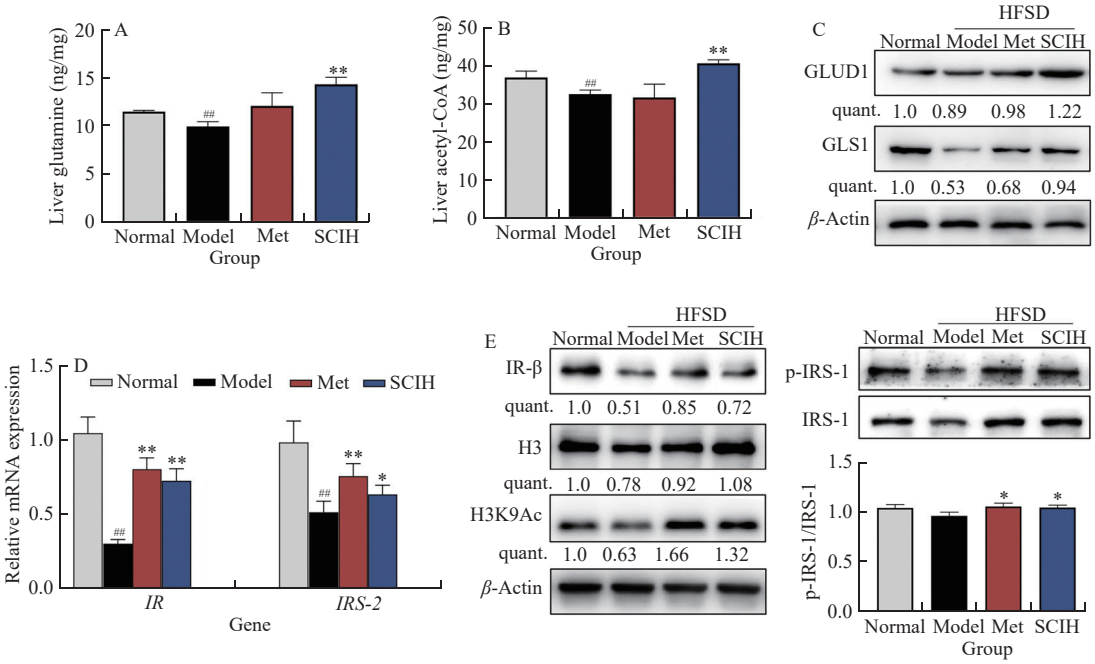

Fig. 7 Effect of SCIH on the glutamine signal in HFSD-induced mice liver (n = 8). (A) Liver glutamine levels. (B) Liver acetyl-CoA levels. (C) Western blot analysis for glutamine metabolism markers of protein expression. (D) mRNA expression of *IR* and *IRS-2*. (E) Protein expressions of IR and IRS-1. Data were expressed as mean ± SEM. $^{\#\#}P < 0.01$ compared with the Normal group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the Model group.

acetylation and directly regulates histone acetylation modification[21]. It has been identified that insulin receptor substrate (IRS) was regulated by histone acetylation[22]. The result exhibited that SCIH supplementation increased the protein levels of histone 3 (H3) and histone 3K9 acetylation (H3K9Ac), thereby elevating the transcription and translation of IRS (Fig. 7E). As is shown in Fig. 7D, the expression levels of *IR* and *IRS-2* genes were remarkedly downregulated in Model group, however, both them were upregulated in SCIH group. Moreover, the IR-β and p-ISR-1/ISR-1 were also significantly increased, indicating that histone acetylation promoted the progression of phosphorylation (Fig. 7E). The above data verified that SCIH participated in the regulation of insulin resistance through acetylation induced by glutamine metabolism.

4. Discussion

Insulin resistance refers to the inability of insulin to function properly. HepG2 cells are widely used to model hepatocytes in glucose metabolism and insulin signaling due to their multiple functions as normal human liver cells[23]. Previous studies reported that HepG2 cell was used to induce insulin resistance state to examine the antidiabetic activity of different compounds[24]. Besides, HepG2 cells were proved to have a glucose metabolism pattern similar to that of the *in vivo* condition[25]. In the current study, we identified sea cucumber intestinal hydrolysates for amelioration of insulin resistance by HepG2/IR model. Over the past few decades, marine active peptides have garnered significant attention from scientists due to their high amino acid content and enriched nutritional properties. The research on the preparation of biological peptides using various enzymatic hydrolysis methods and the verification of their nutritional functions *in vivo* has witnessed a growing trend in contemporary society. Zhu et al. [26] found that low molecular weight marine collagen peptide (< 1 kDa) improved abnormal glucose metabolism and insulin sensitivity in type 2 diabetes mellitus rats. The proteins and peptides in sea cucumber have also been gradually investigated to improve insulin resistance. Previous studies reported the crucial role of certain dominant amino acids, such as glutamate and glycine, in ameliorating chronic metabolic disorders, including insulin resistance[27]. Our results found that glutamic acid was the dominant amino acid in SCIH, and this finding indicated that SCIH had the potential to alleviate insulin resistance.

In recent years, feeding C57BL/6J mice a HFSD for a long period of time (8–20 weeks) has been recognized as the main method for establishing IRMs, and the model has the advantages of being stable, reliable and cost-effective[28]. Thus, we established a model of insulin resistance by diet and investigated the mechanism of SCIH *in vivo*. Patients with insulin resistance often exhibit symptoms of elevated blood glucose levels. The balance between glucose entering circulation and its absorption and metabolism in peripheral tissues determines the concentration of glucose. Homeostasis is maintained through the secretion of insulin by the pancreas, which regulate glucose metabolism and keep postprandial serum glucose levels within the normal range[29]. Sea cucumber hydrolysates could alleviate insulin resistance through reducing serum glucose levels and improving glucose tolerance in diabetic rats[30]. Gong et al.[31] discovered that sea cucumber peptides with a molecular weight less than 3 kDa exhibited the ability to enhance glucose uptake in both 3T3-L1 cells and HepG2 cells through simulating proteolysis process in the digestive tract. The present results showed that the HFSD-induced mice exhibited elevated serum glucose, serum insulin, and GSP levels, while impaired glucose tolerance. Interestingly, dietary SCIH significantly ameliorated fasting serum glucose and the aforementioned biochemical indicators, evidencing that SCIH owned significant glucose regulation ability. From the pathological perspective, we observed that SCIH improved islet vacuolization, repaired the damage of islet microstructure, and facilitated insulin secretion while inhibiting glucagon secretion in islet β-cells. A numerous studies have showed that cytokine-mediated inflammatory responses contribute to apoptosis and dysfunction of pancreatic β-cells, and this dysfunction further affects insulin secretion, leading to the onset and progression of diabetes[32]. Marinho et al.[33] found that a high-fat diet led to an increase in pancreatic inflammatory factors with pancreatic β-cell apoptosis in mice, whereas semaglutide decreased pro-inflammatory markers and restored islet β-cell function to promote insulin secretion. Similar to previous findings, our findings indicated that SCIH reduced the levels of inflammatory factors and increased the insulin secretion in pancreatic tissues, therefore we hypothesized that the effect of SCIH on insulin amelioration might be related to pathways that regulate inflammatory factors.

As mentioned above, insulin resistance is a complex condition that induced by a combination of various factors, including disordered glucose metabolism, lipo-toxicity, and inflammation. The liver is a major target tissue for insulin action and has a more sensitive and critical role in insulin signaling than other tissues[34]. In addition, the liver can compensate for muscle insensitivity to insulin by increasing glycogenolysis and gluconeogenesis[35]. For these reasons, we subsequently explored the mechanisms by which SCIH improves insulin resistance from the perspective of hepatic glucose metabolism. In this study, the liver PAS staining and glycogen assays yielded consistent results, indicating that SCIH supplementation promoted the accumulation of liver glycogen. GK and HK, the key enzymes of intracellular glucose metabolism, regulate the phosphorylation process of glucose and hexose, respectively[36]. Our results proved that SCIH supplementation upregulated the mRNA expression of *GK* and *HK*, which was similar to the apparent indices. The PI3K/AKT pathway is important for regulating muscle glucose transport and liver glycogen synthesis in response to insulin[37]. In the liver, glycogen synthesis is primarily determiner by 2 enzymes, GS and glycogen phosphorylase (GP)[38]. Yang et al.[39] found that a sturgeon protein-derived peptide, KIWHHTF, downregulated GS phosphorylation and PEPCK expression *via* mediating the IRS-1/PI3K/AKT signaling pathway. Gluconeogenesis is another important step in glucose metabolism. According to reports, glucagon is known to enhance the production of glucose by promoting gluconeogenesis. Conversely, insulin operates by opposing the actions of glucagon mainly through the signaling pathway of PI3K/AKT[40]. The transcription factor FOXO1 is well-documented for its activation during fasting and subsequent inactivation after feeding under physiological conditions[41]. Insulin effectively and quickly inhibits hepatic glucose production, and this mechanism is pivotal in achieving this outcome during the postprandial period[42]. Upon insulin resistance, phosphorylated FOXO1 will translocate into the nucleus and bind to PGC-1α, thereby inducing genes expression related to hepatic gluconeogenesis, such as G-6-Pase and PEPCK[14,43]. In the current study, SCIH regulated the protein levels of p-GSK3β and increased glycogen synthesis via promoting the phosphorylation of PI3K and AKT, as well as their downstream cascades in liver, thereby reducing bold glucose concentration and achieving the purpose of alleviating insulin resistance. Furthermore, SCIH decreased the levels of PEPCK and G-6-Pase through FOXO1/PGC-1α signaling pathway,

suggesting that SCIH not only promoted hepatic glycogen but also inhibited gluconeogenesis.

Exogenous glutamate is absorbed through the small intestine, then enters the blood circulation, and is further transported and stored in virous tissues in body. At the same time, tissues can also synthesize glutamine through amino conversion processes, including skeletal muscle, etc. Glutamine plays a crucial role in various nutritional functions within cellular metabolism[44]. In an animal study, chronic oral glutamine supplementation reduced HFD-induced obesity, and improved insulin signaling, together with reversing defects in glucose metabolism[26]. Another study evidenced that glutamine supplementation effectively alleviated insulin resistance through reducing inflammatory factors[45]. GLS is a key rate-limiting enzyme for glutamine metabolism. The amino acid transporter ASCT2/SLC1A5 facilitates the entry of glutamine into the cell, where it undergoes deamination catalyzed by GLS in the mitochondria, resulting in the production of glutamate. Then, glutamate is metabolized into the TCA cycle intermediate α-ketoglutarate (α-KG) through the action of GLUD1. Following the steps of TCA cycle, α-KG is eventually cleaved to acetyl-CoA in the nucleus[46]. From the present results, we observed that SCIH supplementation increased the glutamine and acetyl-CoA content, and upregulated the protein expression of GLS1 and GLUD1 in mice liver. Recently, the role of epigenetics in various intricate disorders, including insulin resistance, has emerged as a pivotal research focus[9]. Combined with our previous studies[9], we found that supplementation with SCIH enriched in glutamate could regulate the glutamine metabolism in cells, while interestingly, the glutamine metabolite acetyl-CoA serves as a crucial metabolic substrate for histone acetylation modification. Based on these findings, we hypothesized that dietary SCIH supplementation may potentially alleviate insulin resistance through modulation of histone acetylation modification.

Studies have demonstrated that the level of intracellular acetyl-CoA is deeply correlated with the level of histone acetylation modification, and the production of acetyl-CoA by specific cellular environments can make acetylation locally driven[47]. Acetylation can modulate a diverse range of enzymes involved in energy metabolism pathways and alter signaling pathways related to metabolism, such as the insulin signaling pathway[4]. H3K9Ac is commonly associated with transcriptional activation of genes and may act by affecting gene expression in the context of insulin resistance. It has been showed that insulin could promote cardiomyocyte glucose uptake through the PI3K-AKT signaling pathway, which might involve changes in H3K9Ac expression[48]. Our previous findings revealed that dietary SCIP induced the elevated expression of pluripotency factors, which in turn exerted biological functions, by up-regulating the expression of histone acetyltransferases and acetylation of the histone H3K9 site. IRS-1, an acetylated protein, is important in the interaction between membrane receptors and intracellular substrates, thereby serving as a pivotal regulator of insulin and IGF signaling pathways[49]. After the binding of IRS-1 protein to insulin receptor, IRS is phosphorylated at the tyrosine site, which leads to the activation of intracellular signals such as PI3K. Notably, histone acetylases induce increased acetylation levels of IRS-1 accompanied by increased tyrosine phosphorylation in response to insulin. Conversely, the interaction between histone deacetylases 2 (HDAC2) and IRS-1 results in deacetylation of IRS-1, leading to decreased tyrosine phosphorylation of IRS[50]. Chriett et al.[51] discovered that when sodium butyrate, an HDAC inhibitor, was given in the diet, it increased insulin sensitivity by increasing the acetylation of histone H3 and upregulating IRS-1 in L6 myotubes. Another study further confirmed that the increase in IRS-1 acetylation induced by trichostatin A was accompanied by an increase in tyrosine phosphorylation on response to insulin, which resulted in a reduction in insulin receptor levels in these cells[52]. Our results exhibited that SCIH supplementation increased the liver acetyl-CoA content, and increased protein expression of H3 and H3K9Ac, thereby promoting the expression of IRS (Fig. 8). Therefore, we suggested that the upregulation of H3K9Ac expression might be

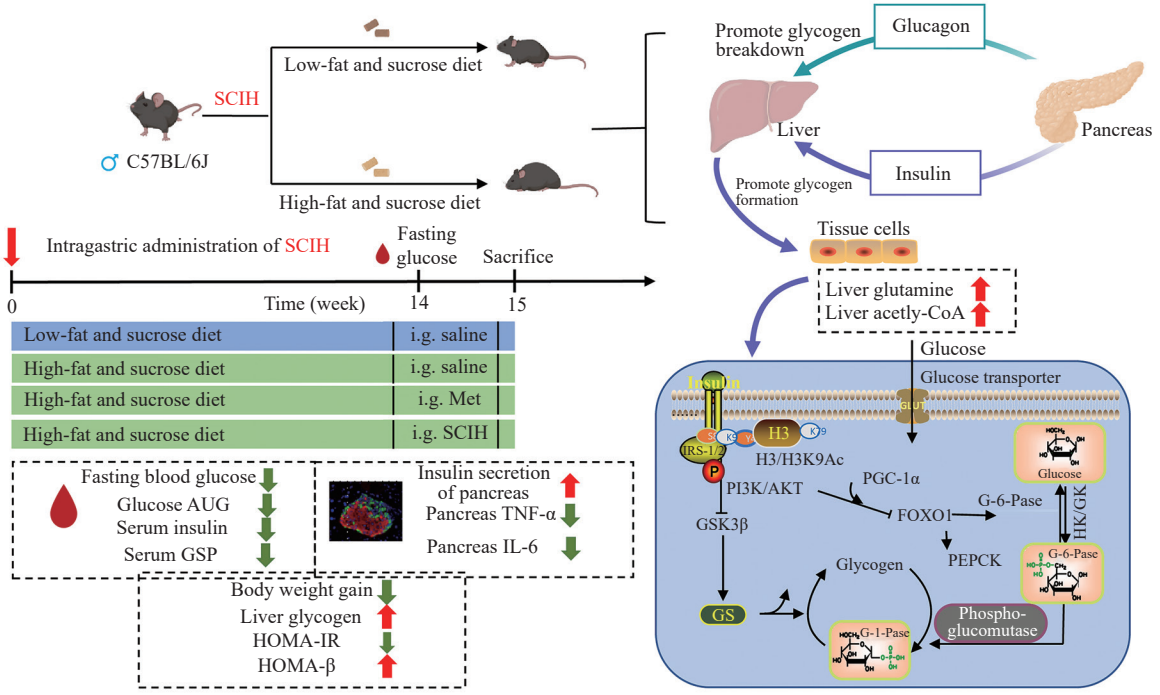

Fig. 8 Possible underlying mechanism on the effects of SCIH in alleviating insulin resistance in HFSD-induced mice.

beneficial and its upregulation might be associated with glutamine metabolism in the current animal model.

However, there are some limitations of the current study, which will be the focus of future research. First, comparative analysis of the amino acid composition in the different enzymatic hydrolysates was not performed. Second, the peptides in the enzymatic hydrolysates were not further isolated, purified and analyzed for identification, thus leading to the current inability to assess the activities of all components and the direct or indirect effects of different peptide sequence compositions on insulin resistance in animal models. Finally, more in-depth studies are needed to explore the mechanism of acetylation modification.

In conclusion, the findings confirmed for the first time that SCIH exhibited insulin resistance activity both *in vitro* and *in vivo*. Specially, the results showed that dietary SCIH not only alleviated insulin resistance through stimulating glycogen synthesis, and suppressing gluconeogenesis in hepatic tissue, but also suggested that the effective regulation of IRS1/PI3K/AKT might be closely related to histone acetylation modification.

Conflicts of competing interest

Yuming Wang is an editorial board member, and Tiantian Zhang is a youth editorial board member for *Food Science and Human Wellness* and wa not involved in the editorial review or the decision to publish this article. The authors declare that they have no competing financial interests and conflicts.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250417.

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