This study investigated the effects of a combination of fucosylated chondroitin sulfate (CHS) and rosiglitazone (RSG) on glucose metabolism in the liver of insulin-resistant C57BL/6J mice fed a high-fat high-sucrose diet for 19 weeks. The results showed that the combination (CHS/RSG) synergistically improved body weight gain, liver weight, fasting blood glucose levels, glucose tolerance on an oral glucose tolerance test, serum insulin levels, homeostasis model assessment indexes, and hepatic glycogen content. In liver tissue, CHS/RSG significantly normalized the activities of hexokinase, pyruvate kinase, and glucose-6-phosphatase. Additionally, it increased the mRNA expression of insulin receptors, insulin receptor substrate 2, phosphatidylinositol 3 kinase (PI3K), protein kinase B (PKB), and glycogen synthase, and inhibited glycogen synthase kinase 3β (GSK-3β) mRNA expression in the liver. This suggests that CHS/RSG treatment improves glucose metabolism by modulating metabolic enzymes and strengthening the PI3K/PKB/GSK-3β signal pathway mediated by insulin at the transcriptional level.

Key words: fucosylated chondroitin sulfate; rosiglitazone; glucose metabolism; glucose metabolic enzymes; insulin signaling

A high calorie diet rich in fat and refined sugar is suggested to be a pivotal factor that accounts for the rising prevalence of insulin resistance and type 2 diabetes. Diets rich in sucrose and fat can produce insulin resistance, and the insulin resistance in the liver precedes resistance in peripheral tissues with high-calorie diets. The pathophysiological repercussions of hepatic insulin resistance include hyperinsulinemia and defects in glycogen synthesis, since the liver is both insulin sensitive and a regulator of glucose-glycogen homeostasis. Glycogen synthesis plays a significant role in glycogenolysis and glucose homeostasis. A significant decrease in glycogenesis occurs in diabetes mellitus due to defective insulin secretion or activity. Xie et al. and Khan et al. found that hepatic glycogen content decreased in insulin-resistant mice. Sulfated polysaccharides from sea cucumbers have attracted considerable attention in recent years. They contain two polysaccharides: sulfated fucan and fucosylated chondroitin sulfate (CHS). As shown in Fig. 1, CHS from sea cucumber is an acid glycosaminoglycan composed of a chondroitin sulfate-E backbone with branches of sulfated fucose at a ratio of approximately 30%, a distinguishing feature different from CHS isolated from other species. Current reports on the bioactivities of this component focus mainly on anticogulation, antithrombosis, and antitumor. But as far as we know, there is not any report on CHS from sea cucumber. CHS isolated from other species has been used as an ingredient in dietary supplements taken as alternative medicine to treat inflammation. Pharmacokinetic studies performed on humans have demonstrated that CHS can be absorbed by the oral route. The structure and molecular size of CHS strongly influence its absorption and bioavailability, higher absorption and bioavailability being reported for lower-molecular-weight CHS. Jin et al. found the dose- and time-dependent transport of disaccharides derived from CHS, though transepithelial transport of CHS was not detectable. In addition, several CHS carriers were used to increase their bioavailability, including chitosan and polyacrylate. Rosiglitazone (RSG) is a widely used thiazolidinedione medication for insulin resistance and type 2 diabetes. However, besides the significantly positive effects on maintenance in glucose homeostasis, RSG administration shown several adverse effects. A dramatic decrease in the use of RSG-containing products

Fig. 1. Chemical Structure of CHS from Sea Cucumber.
has occurred in the United States and Europe,\textsuperscript{23,24} and it will perhaps no longer be approved as monotherapy for type 2 diabetes.\textsuperscript{25} In the present study, we investigated the effects of a combination CHS and RSG on glucose metabolism in HFSD-fed mice. To gain insights into the potential mechanism by which CHS alters the anabolism of glycogen from glucose mediated by insulin in the liver, key enzyme activities of HK, PK, GP, and G6Pase were determined. To verify further the positive influence of CHS on insulin signaling, mRNA expression of key glycogen synthesis-related genes was also measured.

Materials and Methods

Materials. Dried sea cucumber, \textit{Cucumaria frondosa}, was purchased at a seafood market in Qingdao, China, and identified by Professor Yulin Liao of the Chinese Academy of Sciences Institute of Oceanography (Qingdao, China).

Preparation of CHS. CHS was extracted and purified from the body wall of \textit{Cucumaria frondosa} by the method of Chen.\textsuperscript{11} The yield of CHS was about 3.19%. The average molecular weight was 21.53 kDa determined by gel filtration chromatography.\textsuperscript{7} The chemical composition consisted mainly of glucuronic acid, galactosamine, and fucose in a molar ratio of 1/1.14/1.55 analyzed by HPLC following acid hydrolysis and derivatization with PMP (1100, Aglient, Santa Clara, California, USA).\textsuperscript{10} The sulfate content was 27.81% as determined by ion chromatography (ICS-2000, Dionex, Sunnyvale, California, USA).\textsuperscript{11}

Animals and diet. Male C57BL/6J mice, 4–5 weeks, were purchased from Vital River Laboratory Animal Center (Beijing, China; Licensed ID: SCXK2009-0007). They were housed under 12–12 h light-dark condition at 23 ± 1 °C. The use of animals in this study was approved by the Ethics Committee for Experimental Animal Care at Ocean University of China. Type 2 diabetes mellitus model mice were fed a high-fat high-sucrose diet (HFSD, according to the AIN-93 recipe) as describe previously.\textsuperscript{10} The mice were randomized into six groups of 10 animals each: control (low-fat low-sucrose diet, LFSD), HFSD, HFSD + 1 mg of RSG/kg-weight (HFSD + RSG), HFSD + 80 mg of CHS/kg-weight (HFSD + CHS), and HFSD + a combination of CHS and RSG (HFSD + CHS/RSG). The diet composition and CHS content in the experimental diets are summarized in Table 1. After 19 weeks of feeding, blood was collected to test fasting blood glucose level, glucose tolerance, and serum insulin level. The liver was separated to measure glycogen content, glucose metabolism-related enzymes, and gene mRNA expression levels.

Blood glucose parameters. Blood was collected from the tail veins of the mice after a 5-h fasting period. Fasting blood glucose level was determined by a commercial kit (Biosino, Beijing, China). An oral glucose tolerance test was conducted according to blood glucose levels at 0, 0.5, 1, and 2 h after 2 g of glucose/kg-weight intragastrically given. AUC was calculated by formula 1:

\[
\text{AUC} = 0.25 \times A + 0.5 \times B + 0.75 \times C + 0.5 \times D
\]

(A, B, C, and D represent the blood glucose level at 0, 0.5, 1, and 2 h) (formula 1)

Serum insulin level. After 10 h fasting, blood was collected and centrifuged. Serum insulin level was measured with an insulin ELISA kit (Invitrogen, Carlsbad, California, USA). HOMA-IR and QUICKI was calculated formulas 2 and 3, respectively:

\[
\text{HOMA-IR} = \frac{\text{fasting blood glucose level} \times \text{serum insulin level}}{22.5} \quad \text{(formula 2)}
\]

\[
\text{QUICKI} = \frac{1}{\text{lg(fasting blood glucose level)}} + \text{lg(serum insulin level)} \quad \text{(formula 3)}
\]

Hepatic glycogen content. The liver (80–100 mg) was homogenized with alkaline lye and heated 30 min in boiling water. Samples were diluted in 96-fold distilled water and centrifuged at 8,500 rpm for 30 min. The precipitate was dissolved in 1.5 mL of distilled water, and glycogen content was measured with a commercial kit (Biosino, Beijing, China).

Enzymes of glucose metabolism. The activities of key enzymes for glucose metabolism, such as HK, PK, GP, and G6Pase in the liver were assayed by methods outlined elsewhere.\textsuperscript{27}

qRT-PCR analysis. The mRNA expression levels of glycogen synthesis-related gene IR, IRS-2, PTPK, PKB, GSK-3β, and GS were examined by qRT-PCR. Total RNA from the liver was extracted with TRIzol reagent, and 1 μg of RNA was reversed transcribed to cDNA with M-MLV. PCR was tested by amplification of 15 ng the cDNA in 25 μL system containing SYBR Green mix with a quantitative real-time PCR thermocycler (qT5; Bio-Rad, Hercules, California, USA). The PCR cycling conditions were pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and stretching at 72 °C for 30 s through 45 cycles. The primer sequences used in amplification are listed in Table 2. PCR products were quantified by software (iCycler iQ5). The housekeeping gene β-actin was used as internal reference. The mRNA relative expression level was expressed as the ratio of target gene signal intensity to that of β-actin.

Statistical analysis. Results were expressed as mean ± SD with Graph Pad Prism version 3.0 for Windows. One-way analysis of variance (ANOVA) followed by Tukey’s test with SPSS version 17.0 software was used for multiple comparisons. Data were considered statistically significant at \( p < 0.05 \).

Results

CHS/RSG decreased body weight gain and liver weight

As shown in Table 3, though food intake was higher in the HFSD mice than in LFSD mice, calorie intake was unchanged. This indicates that the animals in the present study were under equal calorie circumstances, even if the diet calorie was different. HFSD caused significant increases in body weight gain and in liver weight. RSG and CHS alone significantly lowered liver weight, and the combination (CHS/RSG) further reduced liver weight. CHS and CHS/RSG treatment decreased body weight gain, but the RSG group showed an increase in body weight. The results indicate that CHS/RSG can synergistically decrease liver weight, without the side effect, weight gain, of RSG.
Fig. 2. Effects of CHS/RSG on Oral Glucose Tolerance in the Insulin-Resistant Mice.
A. Blood glucose levels at 0, 0.5, 1, and 2 h after intragastrically administration of 2 g of glucose/kg-weight; B. AUC in the oral glucose tolerance test. Data are mean ± SD value (n = 10). *p < 0.05, **p < 0.01 as compared to control mice; #p < 0.05, ##p < 0.01 as compared to HFSD mice; $p < 0.05, $$$p < 0.01 as compared to RSG-treated mice; $p < 0.05, $$$p < 0.01 as compared to CHS-treated mice.

CHS/RSG decreased blood glucose level
Figure 2 and Table 3 indicated the blood glucose level of the HFSD C57BL/6J mice. HFSD caused a significant increase in fasting blood glucose level and AUC. RSG or CHS treatment remarkably reduced the two blood glucose parameters, and treatment with the combination further significantly tended towards normality. This indicates that CHS/RSG can synergistically decrease blood glucose level.

CHS/RSG improved insulin resistance
Hyperglycemia and hyperinsulinism are the major assessment criteria for type 2 diabetic mellitus. As shown in Table 3, serum insulin level increased significantly in the HFSD group. RSG and CHS treatment showed significant reductions in serum insulin levels and HOMA-IR score, and an increase in QUICKI value, whereas CHS/RSG normalized these parameters better than the individual compounds.

CHS/RSG increased hepatic glycogen content
As shown in Table 3, fed RSG and CHS, hepatic glycogen content remarkably increase by 251.39% and 192.13% relative to the HFSD group, respectively. Hepatic glycogen content also improved more significantly under combination treatment than the lone treatments.

CHS/RSG normalized activities of glucose metabolism-related enzymes
Figure 3 summarises the activities of key enzymes for glucose metabolism in the liver. HK and PK activities decreased, while GP and G6Pase increased in the HFSD mice. In the RSG and CHS treated insulin-resistant mice, these enzymes activities were remarkably restored to near-control levels. The combination supplementation further brought the activities of these enzymes towards normality except for GP. These results suggest that CHS/RSG can synergistically normalize the activities of pivotal enzymes for glucose metabolism to maintain glucose homeostasis.

CHS/RSG regulated mRNA expression of genes
Glycogen synthesis is mainly regulated by the PI3K/PKB/GSK-3β signal pathway mediated by insulin. As shown in Fig. 4, IR, IRS-2, PI3K, and PKB mRNA
expression levels were significantly decreased in the HFSD group. Treatment with RSG or CHS alone brought the above changes towards normality, and the combination treatment further significantly tended towards normality. GSK-3β is a negatively regulated gene in the process of glycogen synthesis. It regulates the activation of GS. When supplemented with RSG or CHS alone, the GSK-3β mRNA expression level downregulated and GS mRNA expression level upregulated in the liver of the insulin-resistant mice. The combination treatment further normalized the mRNA expression levels of the two genes. These results suggest that CHS/RSG can promote hepatic glycogen synthesis by regulating glycogen synthesis-related gene expression.

Discussion

In the present study, we found that a combination of CHS and RSG significantly decreased body weight gain, liver weight, blood glucose level, serum insulin level, and also increased hepatic glycogen content. Moreover, the combination exhibited a synergistic effect on these
therapeutic activities. These findings indicate that CHS can decrease blood glucose levels by promoting glycogen synthesis in the livers of diabetic mice.

The liver plays important roles in maintaining glucose homeostasis. In insulin resistance, hepatic glucose production is unregulated by increased gluconeogenesis and depleted glycolysis.28) Hepatic HK and PK have major effects on glucose homeostasis, and are potential targets in the pharmacological treatment of insulin resistance. Increases in HK and PK activities can cause increased utilization of blood glucose for energy production or glycogen reserves in the liver.29) GP and G6Pase catalyze enzymatic reaction that is included in the gluconeogenesis reactions, and confers on the liver the capacity to release glucose into the blood.20) Decreased HK and PK activities and increased GP and G6Pase activities have been confirmed in several animal models of insulin resistance and type 2 diabetes.30,31) In the present study, simultaneous activation of enzymes that decreased glucose generation (GP and G6Pase) and reductions in those involved in glucose utilization (HK and PK) reduced hepatic glucose output, causing improvements in hyperglycemia and insulin resistance in the CHS- and RSG-treated mice. CHS/RSG failed to change GP activities as compared with single treatment, while the combination synergistically normalized HK, PK, and G6Pase activities.

Glycogen synthesis in the liver is an important mechanism involved in blood glucose homeostasis and metabolism, which is mediated by insulin. Insulin stimulates hepatic glycogen synthesis by activating a complex cascade of signaling pathways.32) Phosphorylation of IR by insulin activates the phosphorylation of tyrosine residues of IRS-2,28) which then activates the downstream proteins PI3K and PKB.33) These are the pivotal protein kinases, and negatively regulate GSK-3β activity.34) Bhuvaneswari et al. have provided evidence that promotion of phosphorylated IR and IRS-2 can decrease hepatic glucose production and hepatic insulin resistance.35) Matveyenko et al. found that decreased hepatic IRS-2 signaling can impaired the activation of downstream insulin signaling effector molecules AKT and Foxo1, and decreased the expression of HK.36) Thus IR and IRS-2 genes are essential for insulin signaling. In this study, CHS treatment alone increased IR and IRS-2 mRNA expression levels by 62.6% and 47.7% respectively in the liver of the HFSD mice (Fig. 4). These increases were strengthened by the combination of CHS and RSG. Inhibition of the PI3K enzyme with pharmacological inhibitors such as wortmanin completely blocks this signal pathway.37) The PKB/Akt inhibitor, MK-2206, effectively inhibits insulin-stimulated glycogen synthesis in rat skeletal muscle.38) Arcari et al. have reported that HFSD downregulated PKB, IRS-2, and PI3K mRNA expression.38) Fan et al. found that the phosphorylation levels of p85-PI3K and PKB decreased in the liver of the insulin-resistant rats.39) In the present study, CHS and RSG treatment significantly increased the mRNA expression levels of PI3K and PKB genes in the liver of the HFSD mice, and these effects were further enhanced by CHS/RSG. These results indicate that CHS/RSG synergistically maintained glucose homeostasis in the liver by activating the PI3K/PKB signal pathway mediated by insulin.

GSK-3β and GS are pivotal enzymes in the PI3K/PKB/GSK-3β signal pathway. GSK-3β is regulated by the activation of PI3K and PKB. Delarue et al. found that activated PI3K and PKB lowered the GSK-3β expression level.40) On the contrary, low-expressed PKB can stimulate overexpression of the GSK-3β mRNA and protein.41) In the present study, CHS and RSG treatment only decreased GSK-3β expression levels by 39.5% and 32.9% respectively (Fig. 4), and the combination administration further reduced it by 21.3% relative to RSG treatment alone. GS was activated by deactivation of GSK-3β, which lies in upstream of GS, in response to insulin.42) Coghlan et al. have reported that selective cell-permeable GSK-3β inhibitors can enhance GS activity.43) Thus GS activity is negatively regulated by that of GSK-3β, and activated GS increases glycogen content.44) Haseena et al. have reported that reduced GSK-3β mRNA expression increased the glycogen content in the liver of type 2 diabetic rats.45) Fang et al. found that activating GS promoted hepatocyte glycogen synthesis both in vivo and in vitro.46) In this study, CHS treatment increased GS mRNA expression level by 60.6% in the liver of type 2 diabetic mice, and the combination further brought key gene mRNA expression towards normality. These results indicate that CHS and RSG synergistically facilitated hepatic glycogen synthesis by regulating the expression of the GSK-3β and GS genes at the level of transcription. Insulin is the key hormone in inhibition of hepatic glucose production. In type 2 diabetes, the liver tissue becomes less responsive or resistant to insulin, and insulin modulates the PKB/GSK-3β signal pathway. In the present study, serum insulin level and insulin resistance were decreased by CHS/RSG signal pathway. The high molecular weight and negative charge of CHS are thought to be factors that suppress the transepithelial transport of CHS. Jin et al. found that the disaccharides of CHS, the smallest units of CHS, were paracellularly absorbed in the intestines.47) A pharmacokinetics study found that a total absorption of 2.5–5% (mean about 3.5%) CHS occurred in 20 human volunteers orally administered 4,000 mg of CHS, while a maximum increase of about 20% of 6-sulfated disaccharide (D16s) was observed.48) A maximum D16s presence in plasma of 0.9–1.3 μg/mL is found.49) Additionally, due to its anionic properties, absorbed CHS can interact with several blood components, in particular with endothelium, which has been reported to have the power to remove approximately 80% of heparin from circulation after administration.50) Absorbed CHS reaching the plasma compartment can be taken up rapidly by the liver cells, where polysaccharides are recovered in rats.51) In the present study, we proved that CHS isolated from sea cucumber, with a structure different from CHS from other species, decreased blood glucose levels through promotion of hepatic glycogen synthesis, which
have to do with the micromolecular metabolite of CHS after digestion of relevant enzymes in the intestine. Hence we intend to investigate the bioavailability of CHS with a specific structure after administration.

In summary, the present study indicates that the combination of CHS and RSG more strongly improved glucose metabolism than CHS or RSG alone. Normalized regulation of key carbohydrate metabolism-related enzymes activities and activation of the PI3K/PKB/GSK-3β insulin signaling cascade were the underlying mechanism of the beneficial effects of CHS/RSG on the promotion of hepatic glycogen reserves in the insulin-resistant mice. Therefore, dietary supplementation with CHS synergistically enhanced the therapeutic effects of RSG, which indicates that the dosage of RSG can be decreased with the assistance of CHS in insulin resistance and type 2 diabetic treatments in the future.

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