Dietary sucrose, sucralose and probiotic sweetener isomaltooligosaccharide divergently affect exercise induced weight loss and its associated skeletal muscle metabolism

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Abstract

Dietary sweeteners are highly favored by obese people who desire to lose weight and have been considered beneficial for weight maintenance and glucose control, but little is known whether they could affect exercise induced weight loss efficacy and its associated perturbations in skeletal muscles, a key organ playing critical roles glucose homeostasis. In the present study, we found daily drinking sucrose- and sucralose-sweetened water failed to influence the effect of exercise on weight reduction of high-fat-high-sucrose (HFHF) induced obese mice, but remarkably attenuated exercise induced improvements on skeletal muscle morphology, anti-fatigue capacity and glucose metabolism. Probiotic sweetener isomaltooligosaccharide (IMO) augmented beneficial effects of exercise on improving glucose metabolism and skeletal muscle morphology. Skeletal muscle transcriptomics analysis revealed that exercise regulated genes involved in fatty acid metabolism, insulin resistance, inflammation and mTOR signaling pathways. Drinking sucrose and sucralose increased mRNA expressions of Yap, IL-6, TNF-α, Fis1, Cytc, Myh2, Myh4, and protein expressions of IL-6, mTOR, FABP4 while lowered HIF1α, demonstrating their detrimental effects on counteracting exercise induced improvements in muscular functions and fatty acid metabolism. Consistent results were also validated in C2C12 skeletal muscle cells *in vitro*. Notably, we found that the exercised mice may be resistant to the excessive weight regain and its impaired glucose metabolism. For the first time, our study proved that both sucrose and sucralose could attenuate protection of exercise against muscular inflammation during the weight regain following weight loss. Collectively, our results reinforce that daily drinking of sucrose and sucralose-sweetened water counteracted exercise induced improvements on skeletal muscle metabolism and could be dietary factors favouring weight regain, while IMO to some extent synergistically augmented such benefits.

1 Introduction

The global obesity epidemic continues its relentless advance, currently affecting billions of population globally[1]. Pathophysiologic changes occur in multiple organ systems due to obesity and are associated with various metabolic derangements and disease processes[2]. Skeletal muscle is essential for metabolic homeostasis[3,4]. Accumulating evidence has revealed obesity induced detrimental effects on skeletal muscle glucose metabolism, such as reduced insulin-mediated glucose uptake and oxidative metabolism. Although mechanism remain unclear, exercise is one of the most effective ways to combat obesity[5–7], and has shown to prevent and mitigate inflammation, glucose and lipid metabolism disorders, mitochondrial dysfunction as well as oxidative stress in skeletal muscles, of which were tightly associated with obesity.

It is noteworthy that diet could greatly affect the effectiveness of exercise on weight control, alter physiological responses to exercise and trigger muscular adaptations via multiple pathways[8,9]. Consumption of simple and low-quality carbohydrates, e.g., sugar, has a direct implication on the physical pathophysiology[10] and excessive intake of sucrose increases the caloric value of the diet and, consequently leads to the development of the global pandemic of obesity[11,12]. In a resent randomized

controlled trial, Thomsen et al., found that dietary carbohydrate restriction augmented weight loss-induced improvements in glycaemia control and liver fat in individuals with type 2 diabetes[13]. The strict control on simple carbohydrates and sucrose intake is essential for weight maintenance[14,15].

To limit the sugar supply in daily foods, non-calorie and low-calorie sweeteners (including low glycemic functional oligosaccharides) are increasingly prevalent in the food supply and their consumption has increased in recent decades[16]. Recent data show that above 25% of children and 40% of adults in the United States consume foods and beverages containing sweeteners, or often added to tea and coffee as sugar substitutes. Although generally regarded as safe, some concerns have been raised about the impacts of the sweeteners consumption on metabolic homeostasis, chronic conditions and risk factors of metabolic diseases, which remain largely unexplored[17]. Notably, no study to the best of our knowledge, has compared the impacts of sucrose versus sweeteners on exercise induced weight loss efficacy and extensively investigated how they could modulate metabolic pathways in skeletal muscle biology (i.e., the core tissue tightly related with exercise-inducible obesity regulation) pertaining to the mechanisms that drive weight loss. Moreover, there is extremely limited evidence on whether consuming the dietary sucralose and sucrose-sweetened water during exercise may influence the weight regain following weight loss, a serious challenge in long-term weight management[18,19].

In the present study, we comprehensively investigated the impact of intake of dietary sucralose and the prebiotic isomaltooligosaccharide (IMO), the two most widely used sweeteners in both foods and beverages applications, dietary supplements and personal care products, as well as sucrose-sweetened water on exercise induced weight loss efficacy and metabolic status. We also investigated impacts of dietary sucrose, sucralose and IMO on key biological pathways involved in exercise-inducible weight loss that were identified using the skeletal muscle transcriptomics. We further explored whether consuming sweeteners or sucrose during exercise could affect skeletal muscle functions in mice underwent weight regain. Our study provides novel insights on comparing metabolic outcomes of consuming sucralose, IMO or sucrose-sweetened water during exercise, offering implications in long-term weight managements.

2 Materials and methods

2.1 Animal study

Healthy male C57BL/6J mice (n = 105, weight ranged 15–18 g) were purchased from Animal Experimental Center of Shaanxi normal university (Xi'an, China). The cages were placed in a standardized laboratory of animals on a 12-h light/dark cycle with controlled room temperature (22 ± 2 ◦C) and humidity (60%). After one week of acclimatization feeding, mice were randomly assigned to normal control (standard chow, NC, n = 15) and high-fat diet and fructose-sweetened water with 20% (HFHF, n = 90), respectively, for 8 weeks. The ingredients of NC were 40% corn flour, 26% wheat flour, 10% bran, 10% fish meal, 10% bean cake, 2% mineral, 1% coarse grains, and 1% vitamin (purchased from Qianmin Feed Factory, Jiangsu, China). The high fat diet contained 60% fat, 20% carbohydrates and 20% protein. The

animals received food and water ad libitum. The body weight and food intake of the animals were measured weekly (Fig. S1).

After the 8-week HFHF feeding, 90 mice that were fed with HFHF showed substantially higher body weight than those fed with NC, and were then randomly assigned to the aerobic exercise training (EX, n = 60) or the group following the change of daily diet from HFHF to normal chow (Diet-replacement, n = 15) without exercise training. Mice underwent exercise training were fed with fed with normal chow and had free access to tap water (EX + W, n = 15), or water supplemented with 0.01% sucralose (EX + TGS, n = 15), 0.4% IMO (EX + IMO, n = 15) and 10% sucrose (EX + SUG, n = 15). The administered dose for sucralose were well below the reported toxic dose, i.e., 16 g per kg body weight [20,21]. Doses for sucrose and sweeteners were selected to make their sweetness comparable. The remaining 15 mice continued with the HFHF. Mice underwent exercise training were trained for three days on an acclimatized running platform with a speed of 5 m/min, 30 min/day, and an incline of 0°. Exercise was conducted 5 days per week and lasted for 3 weeks. Mice were trained in the mid-afternoon (between 6–9 pm).

During the weight loss intervention, all mice were weighed every 5 days and food intake were recorded. At the end of the weight-loss interventions, 7 mice per group were sacrificed. Mice were fasted for one night prior to euthanasia. Blood samples were centrifuged at 4°C, 1000 rpm for 10 min to obtain the serum sample. Quadriceps femoris muscle samples were carefully collected and stored at -80°C until analysis.

To explore the influence of continues consumptions of sweeteners and sucrose on weight regain, the remaining eight mice in each group were re-exposed to HFHF for another four weeks to assess the rate of weight regain. Body weight was measured every two days, and food intake was recorded daily. These mice were sacrificed, and serum samples and muscle tissues were collected, as mentioned above.

The animal experiments carried out in this study conformed to the Guide for the Care and Use of Laboratory Animals, Eighth Edition, ISBN-10: 0-309-15396-4. All animals in the study were approved by the Animal Ethics Committee of Shaanxi Normal University, Xi'an, China.

2.2 Oral glucose tolerance test and insulin sensitivity test

The oral glucose tolerance test (OGTT) and insulin sensitivity test (IST) were performed before sacrificing the animals according to the protocol published elsewhere[22]. After 8h fasting, all animals were given an oral gavage of glucose (1.5 g per kg body weight) or an intraperitoneal injection of insulin (1.0 unit per kg body weight). Blood glucose concentrations were measured by tail clipping at 0, 15, 30, 60, 90, and 120 minutes after glucose administration or insulin stimulation, and the area under curve (AUC) was calculated.

2.3 Fatigue resistance test and muscle glycogen measurement

The fatigue resistance test was performed using the KW-6C Mouse Rotating Bar Fatigue Apparatus (Nanjing Calvin Biotechnology Co., Ltd.). Mice were subjected to a rotational speed of 40 rpm and the

time and distance for which the animals were able to maintain this speed were recorded. Muscle glycogen was measured using commercial kits (Jiancheng Biotechnology Ltd., Nanjing, China, A043-1-1) following the manufactures' instructions.

2.4 Hematoxylin and eosin staining

The Hematoxylin and eosin staining (H&E) staining was applied to determine the effects of exercise with and without consumption of sweeteners or sucrose contained water on the morphology of the skeletal muscle during weight loss and the follow-up weight regain, respectively. The paraffin block quadriceps muscle was cut thinly (5-μm thickness) and was dried at 60°C. H&E staining was performed at room temperature to localize the myocyte nuclei and geometry[22]. The myocyte number and geometry were measured on multiple sections of the quadriceps using Image-Pro Plus 6.0 software.

2.5 Skeletal muscle transcriptome

Total RNA of skeletal muscle was extracted from using TRIzol® Reagent according the manufacturer's instructions. Then RNA quality was determined by 5300 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA sample (OD260/280 = 1.8 ~ 2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, > 1μg) was used to construct sequencing library.

RNA purification and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). The RNA-seq transcriptome library was prepared following Illumina® Stranded mRNA Prep, Ligation from Illumina (San Diego, CA) using 1μg of total RNA. Shortly, messenger RNA was isolated according to polyA selection method by oligo (dT) beads and then fragmented by fragmentation buffer firstly. Secondly double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). The synthesized cDNA was then subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantified by Qubit 4.0, paired-end RNA-seq sequencing library was sequenced with the NovaSeq 6000 sequencer (2 × 150bp read length). The raw paired end reads were trimmed and quality controlled by fastp with default parameters. Then clean reads were separately aligned to reference genome with orientation mode using HISAT2 software. The mapped reads of each sample were assembled by StringTie in a reference-based approach. The RNA-seq sequencing data that support the findings of this study have been also deposited in the CNSA of CNGBdb with accession number 1030528.

2.6 Western Blot Analysis

Frozen quadriceps femoris muscle tissues (~ 40mg) were homogenized with tissue lysis buffer containing protease and phosphatase inhibitor (Beyotime Company, Shanghai, China) in a homogenizer (Proteintech, China). The homogenate was centrifuged at 12000 g for 20 min at 4°C, and the supernatant was collected as tissue sample lysate. After determining total protein by the BCA protein assay kit, equal amounts of protein for each group was separated by precast 10% stain-free gels (Bio-Rad, USA),

transferred to a poly (vinylidene difluoride) membrane, and blocked in 5% nonfat milk. Protein bands were visualized with HRP-labeled Goat Anti-Rabbit IgG antibodies (Beyotime Company, A0208, 1:2000) using the Pierce ECL (Mishushengwu, Xi'an, China). Azure 300 Chemiluminescence Imaging System (Azure Biosystems, USA) was used for blots quantified. The sum of the integrated optical density was obtained, and the mean value was calculated by the software Image J (National Institutes of Health, USA). The primary antibodies used in western blot were as follows: IL-6 (AF7236, 1:1000), mTOR (AF1648, 1:1000), FABP4 (AF6843, 1:1000) and HIF1α (AF7087, 1:1000) from Beyotime and GAPDH (10494-1-AP, 1:5000) from Proteintech.

2.7 Quantitative real-time PCR

Total tissues RNA was extracted with Trizol reagent (Mei5 Biotechnology Co.,Ltd, Beijing, China), according to method described by Instruction manuals. Reverse transcription to cDNA was performed using the high-capacity cDNA kit (Mei5 Biotechnology Co.,Ltd, Beijing China). Gene expression was evaluated by qRT-PCR. Using Hiper SYBR Premix (Mei5 Biotechnology Co.,Ltd, Beijing China) as fluorescent dye. Primer sequences are shown in Stable. Gene expression quantification was carried out using the *GADPH* gene as internal control. Gene expression changes were analyzed using the 2 − ΔΔCt method. The primer sequences are listed in Table 1.

Table 1
Sequences of Primers for RT-qPCR.

Genes	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
Akt	CATGCAGCACCGGTTCTTTG	TAGGAGAACTTGATCAGGCGG
AMPK	TCTCTATGCTTTGCTGTGTGGAACC	GAGGTGGTAGGCGACGGCTAG
Cytc	AATCTCCACGGTCTGTTC	CCCTTTCTCCCTTCTTCTT
Fis1	GGAACGCCTGATTGATAA	GATTTGGACTTGGAGACA
IL-6	TGATGGATGCTACCAAACTGGA	GTGACTCCAGCTTATCTCTTGG
mTOR	ATCCTGACCCTGATATCCGCT	GGCTGGGTTCATGCTGCTTA
Myh2	TCTCTGATAACGCCTACC	CCTCCTCCTTCTTCTTGT
Myh4	TCTCTGATAACGCCTACC	CCTCCTCCTTCTTCTTGT
PI3K	GCGTGACATGTAGGCTCTCAG	CACACCCCAGCCAATCAAGT
TNF-α	CCCTCACACTCACAAACCAC	ACAAGGTACAACCCATCGGC
Yap	TTCGGCAGGCAATACGGAAT	ATTCGGAGTCCCTCCATCCT
GAPDH	TCACCATCTTCCAGGAGCGAGAC	TGAGCCCTTCCACAATGCCAAAG

2.8 Cells

C2C12 cells were purchased from the Procell Life Science&Technology Co.,Ltd (CL-0044, Wuhan, China). C2C12 cells were cultured in a cellular incubator with 5% CO_2 and kept at 37°C. The complete medium consisted of high-sugar DMEM, 10% FBS, 1% penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Cells were treated with recombinant palmitic acid (1 mM, 24h; MedChemExpress), Acetyl-L-carnitine hydrochloride (1 mM, 1h; MedChemExpress), with and without IMO (50 mM, 3h) and TGS (50 mM, 3h), respectively. The concentrations of IMO and TGS added in the culture medium were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and the cell viability were > 80%.

After the induction of differentiation, cells were stained with Oil Red O (60% isopropanol and 40% water). Briefly, the cells were gently washed with phosphate-buffered saline (PBS) and stained with filtered Oil Red O solution for 30 min. After staining, the Oil Red O staining solution was removed, and the plates were rinsed with water and then dried. The stained lipid droplets were viewed on an Olympus microscope (Olympus, Tokyo, Japan). To analyze the contents of the cellular triglycerides (TG) and total cholesterol (TC), the cells were washed with PBS, scraped into 200 μL of PBS, and sonicated for 1 min. The lysates were assayed for their triglyceride and total cholesterol content using assay kits from Jiancheng Biotechnology Ltd., (Nanjing, China, A110-1-1).

2.9 Statistical analysis

R software (V4.2.2) and GraphPad Prism 8 were used to conducted statistical analyses. Experimental data were shown as means ± SD (standard deviation) for continuous variables.

For transcriptomics data, we applied linear models for microarray and RNA-seq data (R packages “limma”) to identify differentially expressed genes (DEGs) between NC, EX or Diet-replacement group with HFHF group, respectively. Significance was determined by |logFC|>1 and P value < 0.05. We performed GSEA (Gene Set Enrichment Analysis) pathway enrichment analysis to clarify the biological functionality differed between NC, EX or Dite-replacement group with HFHF group (R packages “ClusterProfile”). The significantly abundant pathways were identified by P value < 0.05. To identify key biological pathways involved in exercise- inducible weight loss in muscle development and function, we constructed an interplay network using microarray data from the transcriptome and analyzed the network to identify highly connected genes and gene clusters.

3 Results

3.1 Sucrose, TGS and IMO unequally affect glucose metabolism, anti-fatigue capacity and skeletal muscle morphology during exercise-induced weight loss

The 8-week HFHF feeding substantially increased body weight of mice by 62% than those fed with NC (Fig. 1a, b). HFHF also increased fasting blood glucose, detrimentally affected glucose tolerance and insulin resistance, as evidenced by the significant increases in AUCs of OGTT and IST (Fig. 1c-g). HFHF negatively impaired the anti-fatigue capacity, as evidenced by the reduction in the running distances and

time stayed spent on the instrument (Fig. 1h, i), alongside lower skeletal muscle mass (Fig. 1m) compared with the NC group. HFHF also increased the muscle glycogen content (Fig. 1l). Histopathological results confirmed that HFHF reduced the fiber cross-section area of quadriceps muscle while increased the inflammatory infiltrate, compared with NC group (Fig. 1j, k).

The 3-week exercise reduced body weight, improved glucose tolerance, insulin sensitivity and anti-fatigue activity, successfully alleviated inflammatory infiltration in skeletal muscle while improved muscle fiber cross-sectional area of HFHF-induced obese mice (Fig. 1j, k). Notably, consuming sucrose, TGS or IMO-contained water during exercise had no impact on the exercise induced weight loss efficacy, but differently affected physiological indicators, independent of food and water intake. Specifically, IMO augmented beneficial effects of exercise on improving glucose metabolism and skeletal muscle morphology, which outperformed other treatments in restoring HFHF-induced muscle loss (Fig. 1h-m). Drinking TGS-sweetened water failed to restore HFHF-caused abnormal losses in muscle mass and fiber cross-section area, while increased the inflammatory infiltrate, compared with mice drinking tap water. Drinking sucrose-sweetened water eliminated exercise induced improvements on anti-fatigue capacity and glucose tolerance.

In addition, we observed that the 3-week Diet-replacement (i.e., changing daily diet from HFHF to normal chow) without any exercise could also reduce body weight of HFHF-induced obese mice (Fig. 1a). However, no benefit was seen for glucose regulation and skeletal muscle functions HFHF-induced obese mice.

3.2 Skeletal muscle transcriptome reveals key biological pathways involved in exercise- induced weight loss

RNA sequencing on skeletal muscle was performed in order to identify the biological pathways and hub genes involved in weight loss. We detected 5713 genes with a fragments per kilobase of exon per million fragments mapped (FPKM) value and qualified annotations in skeletal muscles. Among them, 335 genes were upregulated by HFHF (e.g., Atf5, Elk4, Vegfa) compared with NC, mostly involving in PI3K-AKT pathways, while 305 were downregulated (e.g., Mapk, Ppar, p53, Myh4, Jun, Cyc1 and Yap), which were involved in PI3K-AKT, NF-κB, JAK-STAT and MAPK pathways.

The 3-week exercise intervention remarkably influenced the skeletal muscle transcriptome, and enabled to restore gene profiles that were altered by HFHF, leading to a similar skeletal muscle transcriptome compared with the NC group (Fig. 2a, d, e). In compared with the HFHF group, EX upregulated 143 genes while down-regulated 788 genes (Fig. 2b). DEGs were predominately related to fatty acid metabolism, amino acid metabolism, insulin resistance, and Hippo, AMPK, and mTOR signaling pathways (Fig. 2f, g).

Additionally, DR induced 261 DEGs however, such regulatory effects on a large number of genes in relation with obesity were distinct from exercise (Fig. 2c). For instance, DR uniquely upregulated genes involved in amino acid metabolism, Diet-replacement was not effective to restore gene profiles that were

altered by HFHF, and to some extent unfortunately worsen these abovementioned genes disrupted by HFHF-induced disorder, thereby resulting in the skeletal muscle transcriptome that was substantially distinct from NC. Results from GESA analysis revealed hub genes that may play critical roles in the obesity (i.e., AMPK, MAPK, JAK-STAT) and exercise -induced weight loss, including MAPK, PPAR, p53 and Myh4.

3.3 Sucrose, TGS and IMO unequally affect muscular function and transcriptomics-identified pathways related with exercise-induced weight loss

We hypothesize that sucrose, TGS and IMO may cause varied impacts on exercise induced regulation on skeletal muscle transcriptome, which could explain the observed differential influences on glucose metabolism, anti-fatigue capacity and skeletal muscle morphology during weight loss. We then assessed the expressions of transcript genes and proteins in skeletal muscle that were related with HFHF-induced obesity and were regulated by exercise, as revealed by comparative transcriptomics analysis.

Specifically, as shown in Fig. 3a, compared with NC, HFHF increased mRNA expressions of Yap (Fold change $_{HFHF/NC}$ =3.07), IL-6 (Fold change $_{HFHF/NC}$ =4.63), TNF-α (Fold change $_{HFHF/NC}$ =2.20), Fis1 (Fold change $_{HFHF/NC}$ =3.41), Cytc (Fold change $_{HFHF/NC}$ =2.51), Myh2 (Fold change $_{HFHF/NC}$ =3.05) and Myh4 (Fold change $_{HFHF/NC}$ =3.35). By contrast, HFHF lowered AMPK, PI3K and Akt mRNA expressions relative to NC group (Fig. S2). These findings confirmed the severe inflammation and deteriorated muscle fiber composition induced by HFHF.

Exercise effectively downregulated gene expressions of Yap, IL-6, TNF-α, PI3K, Fis1, Cytc, Myh2 and Myh4 alongside with weight loss (Fig. 3a). Notably, drinking TGS- and sucrose- sweetened water exacerbated inflammation, as evidenced by higher expressions of Yap, IL-6 and TNF-α compared with HFHF ($p < 0.05$). Besides, in comparison with mice drinking tap water, mice drinking TGS- and sucrose- sweetened water showed higher expressions of Fis1, Cyct, Myh2 and Myh4, as well as lower PI3K. Consistently, TGS and sucrose intake eliminated exercise induced reduction in protein expressions of IL-6 (Fig. 3b, c) and FABP4 (Fig. 3b, e).

Moreover, drinking IMO-sweetened water had no additional impacts on mRNA expressions beyond the exercise, except for the synergistic effect with exercise on inhibiting HFHF induced abnormal changes in protein expressions of IL-6 (Fig. 3b, c), mTOR and HIF1α (Fig. 3d, f). Diet-replacement restored HFHF-induced inflammation and muscular dysfunction, but the effect size on regulating the genes and proteins was much weak than EX. In addition, strong correlations between biochemical indicators, proteins and genes were demonstrated.

We further validated distinct effects of sucrose, TGS and IMO on the lipid metabolism in C2C12 skeletal muscle cells. We found that cells treated with palmitic acid had higher TG and TC compared with NC. The addition of ALCAR with and without adding TGS did not influence TG and TC contents, while the addition of IMO reduced TG and TC, compared with HFHF (Fig. 3g, h). The Oil Red O staining results revealed that

AICAR with and without IMO could alleviated the lipid accumulation in palmitic acid treated C2C12 myoblasts (Fig. 3i).

3.4 Sucrose and TGS weaken protection of exercise against weight regain-exacerbated inflammation and muscular dysfunctions

We investigated whether consuming sweeteners or sucrose- sweetened water during exercise may affect metabolic status and skeletal muscle of mice at the following weight regain. As expected, compared with NC, a 4-week follow-up HFHF feeding significantly increased body weight while worsen glucose homeostasis in mice underwent weight loss interventions (Fig. 4a-d).

We observed exercise-treated mice had better anti-fatigue capacity and larger cross-sectional area of skeletal muscle compared with mice continuously fed with HFHF, although no difference in body weight, fasting glucose and glucose tolerance was observed (Fig. 4e-i). The exercise-treated mice showed lower AMPK, IL-6, TNF-α, PI3K, Fis1, Cytc, Yap and Myh4 upon the follow-up HFHF compared with mice continuously fed with HFHF (Fig. 4o, and Fig. S3). Such regulatory effects were eliminated or weaken by TGS- and sucrose-sweetened water. Besides, drinking TGS, sucrose or IMO sweetened water did not influence exercise induced reduction in protein expression of FABP4 during weight regain (Fig. 4j, m). Consuming IMO was found to inhibit the protein expression of IL-6 during the weight regain period (Fig. 4k).

4 Discussion

Dietary sweeteners are widely applied in foods, beverages and even health products for weight loss and weight managements. Despite the well-known benefits of exercise on combating obesity and its related metabolic diseases[23–25], to data, less is known regarding how sweeteners affect weight loss efficacy of exercise and its related metabolic pathways in skeletal muscle biology pertaining to the mechanisms that drive weight loss. The present study provides novel and compelling evidence on differing effects of sweeteners, i.e., the prebiotic sweetener IMO, artificial sweetener TGS, and sucrose consumption during exercise on skeletal muscle functions as well as glucose and lipid metabolism during weight loss in HFHF-induced obese mice. Notably, the choice on daily consumption of sucrose or sweeteners during weight loss regimen was for the first time reported to influence the glucose homeostasis and skeletal muscle functions during the diet-induced weight regain, i.e., the repeated process of losing and gaining weight that may further increase risk for developing diabetes and cardiometabolic diseases[18,26,27].

Skeletal muscle is the predominant site for glucose disposal and lipid metabolism. Harmful impacts of diet induced obesity on skeletal muscle health, including mass loss, muscle strength and structure, as well as metabolic functions, have been demonstrated, which may be reversed by exercise training[28]. In line with previous reports, the 3-week aerobic exercise effectively reduced body weight of HFHF-induced obese mice, improved glucose homeostasis and muscle fiber cross-sectional area. Comparative

transcriptomics analysis on skeletal muscle further demonstrated effects of exercise on restoring HFHF-altered genes involved in several metabolic pathways, e.g., fatty acid metabolism, amino acid metabolism, insulin resistance, apoptosis and inflammation. In agreement with our findings, exercise has been reported to enhance glucose uptake and stimulate fatty acid oxidation in skeletal muscle[29,30]. Exercise also has shown to regulate amino acid metabolism, improve muscle oxidative metabolism and reduced insulin resistance in diet-resistant obese humans and mice[31–33].

Previous studies consistently reported associations between consumption of predominant added sugars in food industry, e.g., sucrose and fructose, or sweeteners (e.g., TGS, acesulfame K, aspartame, and saccharin) with the risk of obesity, dyslipidemia, insulin resistance, and metabolic diseases, independent of total energy intake[34], but little is known regarding their potential impacts on the efficacy of exercise for weight loss. Herein, we found that daily consumption of sucrose contained water during exercise intervention significantly eliminated the efficacy of exercise on weight control and on improving insulin sensitivity, in line with previous study which reported the negative impacts of simple and low-quality carbohydrates on the effectiveness of exercise on weight control[8,9]. Sweeteners are thought to be useful replacements in sweet food and beverages. Although replacing sucrose intake with sweeteners may lead to benefits on glucose and lipid homeostasis stemming from weight management[35,36], it is worthy to note the distinct effects of the prebiotic sweetener IMO and artificial sweetener TGS on biochemical parameters and expressions of genes and proteins involved in key pathways associated with HFHF-induced obesity. IMO intake augmented beneficial effects of exercise on improving glucose metabolism and skeletal muscle functions of HFHF-induced obese mice, while TGS failed to alleviate HFHF-induced skeletal muscle loss and dysfunctions as well as inflammation compared with exercise trained mice supplied with tap water.

Mechanistically, exercise and IMO synergistically activated the PI3K-AKT-mTOR signaling to improve glucose metabolism in skeletal muscle. AKT-PI3K signaling plays an important role in systemic glucose mobilization by promoting GLUT4 expression in skeletal and cardiac muscle cells[37]. It has been shown that diet-induced obesity reduced the expressions of PI3K and AKT in skeletal muscle cells, while exercise effectively promoted glucose utilization and insulin sensitivity in the body and facilitated better glucose uptake by skeletal muscle, in line with our results[38–40]. Moreover, Fis1 and Cytc were biomarkers of inflammation and oxidative stress, inducing mitochondrial-mediated inflammatory response[41], and were elevated in obese mice[42]. As expected, consuming IMO sweetened water and exercise acted synergistically in reducing HFHF-induce inflammation and inhibiting Fis1 and Cytc expression. The opposite effects were seen for drinking sucrose and TGS water. Moreover, the Myh2 and Myh4 are expressed in the fast twitch type 2A fibers and type 2B fibers, which have been closely related with exercise performance and could influence the contraction - relaxation activity in skeletal muscles[43–45]. HFHF caused muscular dysfunction and increased mRNA expressions of Myh2 and Myh4, in line with previous study[42]. Drinking TGS- and sucrose- sweetened water attenuated efficacy of exercise on lowering Fis1, Cytc, Myh2 and Myh4, indicating their antagonistic effects on exercise induced improvements in muscle fiber composition and muscular functions.

Disturbances in fatty acid metabolism are evident in obesity and intricately linked with metabolic inflammation and insulin resistance[4,46]. Fatty acid binding proteins (FABPs) are members of the adipokine family of multifunctional proteins, which play important roles in fatty acid metabolism. FABP4, a typical intracellular lipid chaperone, is responsible for promoting lipid storage, distribution, transportation, decomposition and metabolism[47], which was found to be higher in the adipose tissues of obese diabetic individuals compared with healthy ones[48]. FABP4 is an adipogenic protein involved in lipid histotrophic nutrition and is the target of mTOR and PPAR signaling pathways in a variety of tissues[49]. HIF1α a central regulator of glycolysis during hypoxia, is involved in angiogenesis and lipid metabolism[50], which was shown to be selectively activated by exercise[51]. We found exercise induced a significant reduction in FABP4 expression in skeletal muscle of HFHF-induced obese mice, in line with previous studies[52,53]. But such effects were antagonistically abolished when exercise trained mice were supplied with sucrose or TGS sweetened water. Besides, drinking IMO sweetened water during exercise successfully restored HFHF induced reduction in the protein expression of HIF1α in muscles, indicating that daily drinking of IMO may amplify the beneficial impact of exercise on both metabolic pathways within skeletal muscle and energy homeostasis, which warrants further investigations.

One of the noticeable challenges in the management of obesity is the prevention of weight regain after successful weight loss. This weight recidivism is often attributed to the lack of compliance with weight loss paradigms, such as appropriate food habits and exercise[54,55]. A recent study proved that calorie restriction, one of the most common approaches for obesity treatment, could alleviated the high-fat-diet induced excessive weight regain in mice resuming ad libitum feeding at the post-calorie restriction stage[56]. Here, for the first time, we found that the exercised mice may be resistant to the excessive weight regain and its impaired glucose metabolism. Most importantly, consuming sucrose negatively weaken benefits of exercise on glycemic status and skeletal muscle functions at the following weight regain, independent of food intake. It is known that body weight and fat mass are regulated by numerous physiological mechanisms, including metabolic adaptation, inflammatory responses, adipokine secretion and lipolysis[55]. As expected, drinking sucrose sweetened water increased IL-6, TNF-α and mTOR expressions as well as genes involved in muscular functions (i.e., Myh2, Myh4, Fis1 and Cytc), which partly explain the worse metabolic status at the weight regain stage in exercised mice fed on sucrose.

5 Conclusions

Our study provides novel insights in understanding how caloric sucrose or sweeteners could affect efficacy of exercise on weight control and improved metabolic homeostasis. Drinking water containing TGS (even below the ADI does) or sucrose during exercise counteracted the benefits of exercise on improvements of glucose homeostasis, inflammation status and skeletal muscle metabolisms, while IMO amplified exercise-improved anti-fatigue capacity and metabolic status alongside the weight loss. Moreover, the prior exercise may protect against the metabolic disturbance caused by the weight regain, but such effects could be eliminated by sucrose consumption. Our study collectively reinforces the

necessity in controlling and limiting the use of sugar or dietary sweeteners -sweetened beverages to achieve the maximum weight-loss-efficacy of exercise.

Declarations

CRediT authorship contribution statement

Yuan Liu: Investigation, Date curation, Formal analysis, Visualization, Writing-Original Draft. **Yongchang Duan:** Methodology, Validation. **Tianqi Liu:** Investigation, Date curation, Formal analysis, Visualization. **Baobao Zhang:** Investigation, Methodology. **Wanyu Zhu:** Investigation, Validation. **Jie Kang:** Conceptualization, Supervision. **Kunru Zhang:** Conceptualization, Supervision. **Lin Shi:** Conceptualization, Funding acquisition, Writing-Original Draft, Writing-Review & Editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Data will be made available on request.

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Figures

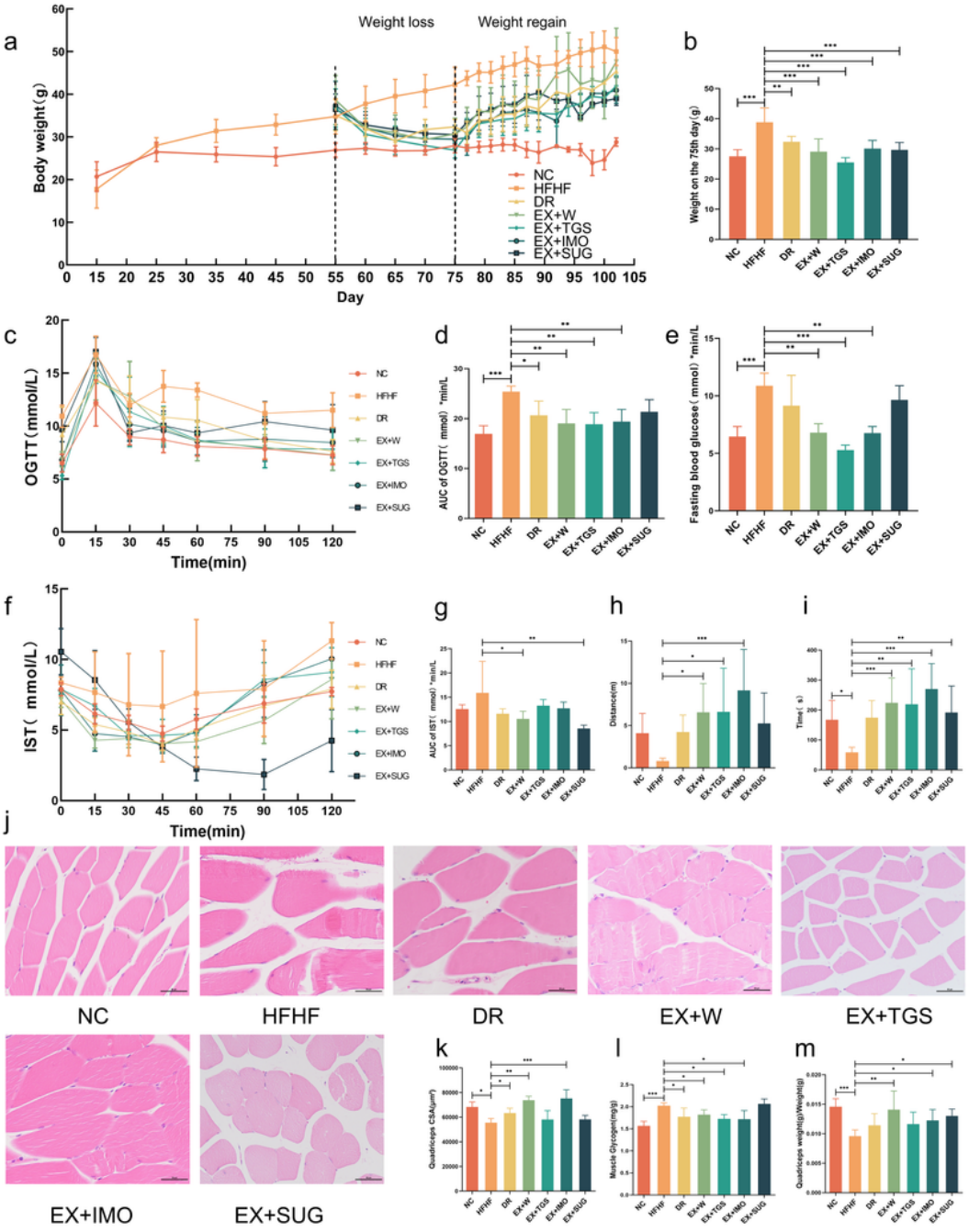

Figure 1

Animal experiment design and effects of sweetener on metabolic status. (a) The measurement of body weight during the intervention. (b) The measurement of body weight after the exercise induced weight loss. (c) Glucose concentrations measured during the oral glucose tolerance test (OGTT). (d) Area under the curve (AUC) of OGTT. (e) Fasting blood glucose. (f) Insulin tolerance test (IST). (g) Area under the curve (AUC) of IST. (h) Running distance measured by the fatigue resistance test. (i) Running time measured by the fatigue resistance test time. (j) H&E staining images of quadriceps femoris muscle tissues (original magnification of 20×); (k) Cross-sectional area of quadriceps femoris muscle. (l) Concentrations of muscle glycogen. (m) quadriceps femoris-to-body weight ratio. Data are presented as mean ± standard deviation (SD). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs the HFHF group. Normal diet, NC (n = 7); high-fat diet and fructose-sweetened water, HFHF (n = 7); change of daily diet from HFHF to normal chow, Diet-replacement (n = 7); exercise and had free access to tap water, EX+W (n = 7); exercise and water supplemented with 0.01% sucralose, EX+TGS (n = 7); exercise and water supplemented with 0.4% IMO, EX+IMO (n = 7); exercise and water supplemented with 10% sucrose, EX+SUG (n = 7).

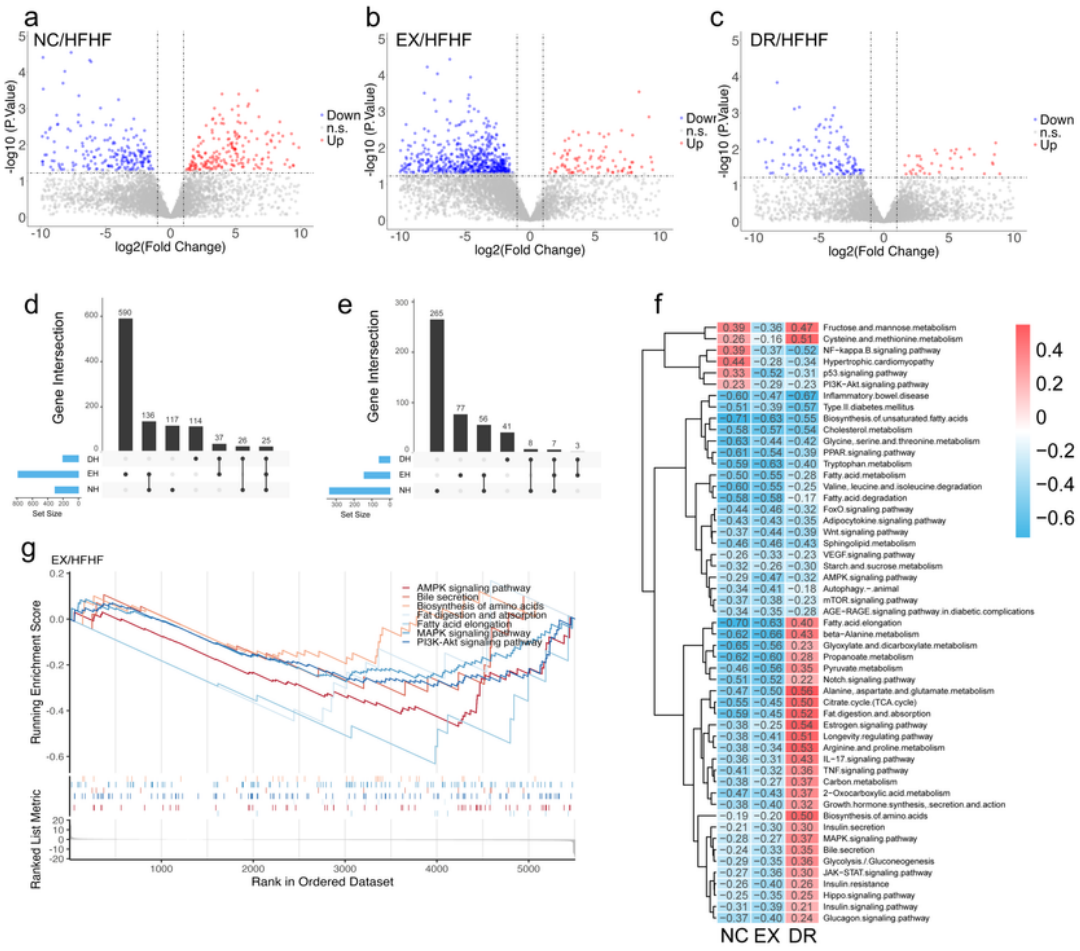

Figure 2

Skeletal muscle transcriptomics analysis reveals key biological pathways involved in exercise- inducible weight loss. (a) Volcano plot presenting DEGs between NC and HFHF. (b) Volcano plot of DEGs between EX and HFHF. (c) Volcano plot presenting DEGs between DR and HFHF. The cut-off criteria were |log2 FC| > 1.5 and adjusted p-value < 0.05. The red dots represented the upregulated genes, and the blue dots represented the down-regulated genes. (d) Gene down-veen of quadriceps in NC, EX, and DR groups. (e)

Gene up-veen of quadriceps in NC, EX, and DR groups. (f) DEGs enrichment analysis. (g) GSEA (Gene Set Enrichment Analysis) pathway enrichment analysis between EX with HFHF group. Normal diet, NC (n = 4); high-fat diet and fructose-sweetened water with 20%, HFHF (n = 4); change of daily diet from HFHF to normal chow, DR (n = 4); exercise and had free access to tap water, EX (n = 4).

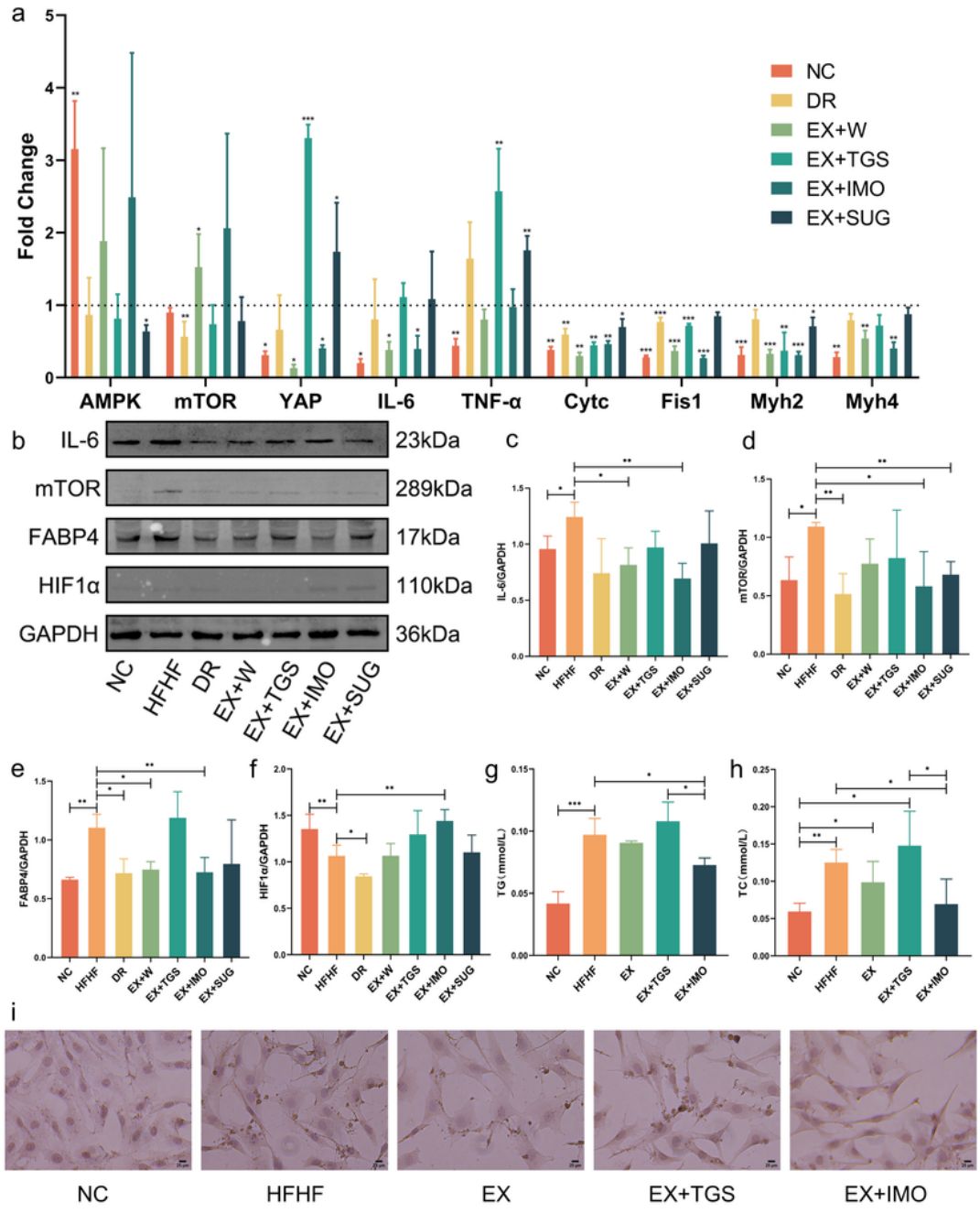

Figure 3

The effects of sucrose, TGS, and IMO on muscle functions and metabolic pathways during exercise. (a) Differences in mRNA expressions of AMPK, mTOR, Yap, IL-6, TNF-α, Cytc, Fis1, Myh2 and Myh4 between treatments and HFHF group. Changes are shown as Ln (fold change HFHF/postbiotics). (b) Western blot analysis on protein expressions of IL-6, mTOR, FABP4 and HIF-1α. (c) Differences in protein expression of IL-6 between groups. (d) Differences in protein expression of mTOR between groups. (e) Differences in protein expression of FABP4 between groups. (f) Differences in protein expression of HIF1α between groups. (g) Total glycerides concentrations in C2C12 cells. (h) Total cholesterol concentrations in C2C12 cells. (i) Oil red O staining images of C2C12 cells (original magnification of 40×). Data are presented as mean ± standard deviation (SD). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs the HFHF group. Normal diet, NC (n = 7); high-fat diet and fructose-sweetened water, HFHF (n = 7); change of daily diet from HFHF to normal chow, Diet-replacement (n = 7); exercise and had free access to tap water, EX+W (n = 7); exercise and water supplemented with 0.01% sucralose, EX+TGS (n = 7); exercise and water supplemented with 0.4% IMO, EX+IMO (n = 7); exercise and water supplemented with 10% sucrose, EX+SUG (n = 7).

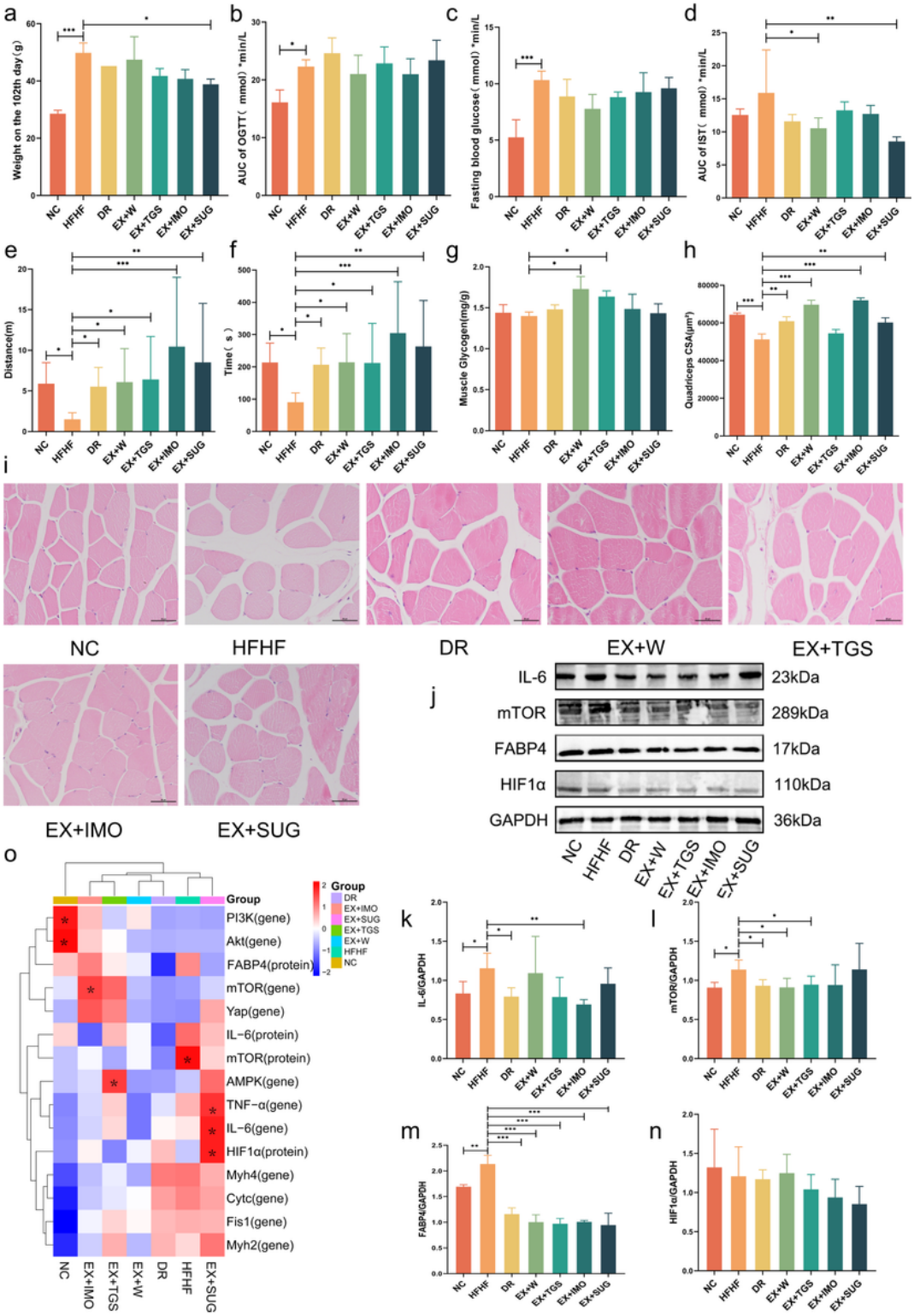

Figure 4

Sweeteners weaken protection of exercise against weight regain-exacerbated inflammation and muscular dysfunctions. (a) Body weight measured after 4-week weight regain; (b) Area under the curve (AUC) of OGTT. (c) Fasting blood glucose. (d) Area under the curve (AUC) of IST. (e) Running distance measured by the fatigue resistance test after weight regain. (f) Running time measured by the fatigue resistance test after weight regain. (g) Levels of muscle glycogen. (h) Cross-sectional area of quadriceps femoris

muscle. (i) H&E staining images of quadriceps femoris muscle tissues (original magnification of 20×). (j) Western blot analysis on protein expressions of IL-6, mTOR, FABP4 and HIF-1α. (k) Differences in protein expression of IL-6 between groups. (l) Differences in protein expression of mTOR between groups. (m) Differences in protein expression of FABP4 between groups. (n) Differences in protein expression of HIF1-α between groups. (o) Correlations between genes, proteins involved in fatty acid metabolism and inflammation in skeletal muscle during weight regain. Data are presented as mean ± standard deviation (SD). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs the HFHF group. Normal diet, NC (n = 8); high-fat diet and fructose-sweetened water, HFHF (n = 8); change of daily diet from HFHF to normal chow, Diet-replacement (n = 8); exercise and had free access to tap water, EX+W (n = 8); exercise and water supplemented with 0.01% sucralose, EX+TGS (n = 8); exercise and water supplemented with 0.4% IMO, EX+IMO (n = 8); exercise and water supplemented with 10% sucrose, EX+SUG (n = 8).

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