Non-digestive stachyose enhances bioavailability of isoflavones for improving hyperlipidemia and hyperglycemia in mice fed with high fat diet

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Abstract

This study examined the efficacy of non-digestive stachyose on enhancing the absorption of soy isoflavones to improve metabolic syndrome in C57/BL6 mice. UPLC-q/TOF-MS was employed to analyze the content of isoflavones in urine and faeces. Stachyose significantly increased urinary contents of total isoflavones, genistein, daidzein and glycitein in mice. Supplementation of stachyose, soybean isoflavones or a combination prevented high fat diet (HFD)-induced body weight gain, accumulated adipose, dyslipidemia and hyperglycemia in obese mice. Interestingly, co-supplementation of stachyose and isoflavones improved all the mentioned parameters more effectively than administration of stachyose or isoflavones alone. Histological observation of hepatic tissues also confirmed the beneficial effects of co-supplementation of stachyose and isoflavones. These findings suggest that co-ingestion of non-digestible oligosaccharides and polyphenols as normal diet is a promising potential strategy for managing or reducing the risk of metabolic syndrome, which will lead to new knowledge on whole soybean and have extensive application in development of healthy food.

Keywords: Enhancing absorption, Hyperlipidemia, Hyperglycemia, Soybean isoflavones, Stachyose

1. Introduction

In our daily life, an excessive consumption of saturated fats is well known to be linked to cardiovascular diseases [1,2], which counts the primary cause of death worldwide [3]. Studies have shown that long-term or excessive intake of high fat diets (HFD) including beef tallow can cause abdominal obesity, dyslipidemia, hyperglycemia, which seems to be the early step in the etiological cascade, leading to obesity and metabolic syndrome [4-7]. It is generally believed that the mechanism of HFD-caused obesity is often associated with lipid metabolism disorders and expanded adipose tissue mass, resulting in high circulating concentrations of free fatty acids (FFAs) [4]. The increased FFAs may lead to the blockage of insulin signal transduction, which can directly contribute to development of insulin resistance (IR) [8]. Importantly, IR is often cited as a potential booster to develop non-alcoholic liver damage, type 2 diabetes, cardiovascular disease and so on, which has become a great threat to human health [9]. What's more, expanded adipose tissue can lead to the increased proinflammatory cytokines, resulting in a chronic low-grade inflammation state and oxidative stress, which are closely related to the pathophysiology of liver damage [10,11]. Despite there have been many chemical medicines for prevention and

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treatment on metabolic syndrome and its associated diseases, more and more evidences have indicated that long-term use of drugs may give rise to resistance and side effects [8]. Fortunately, some natural foods and their bioactive ingredients have been applied to prevention of metabolic syndrome and its complications due to their efficacy and safety [12-15].

Interest in soy foods has increased with the rising awareness of its benefits on health among consumers because of the biological activity of dominant constituents such as stachyose and isoflavones [16,17]. Stachyose is tetrasccharide polymer with two alpha-D-galactosyl residue linked (1 → 6) to the D-glucosyl residue of sucrose (Fig. 1A). It is an excellent dietary fiber and prebiotic, and also is one of the best known and most commonly applied oligosaccharides, which is widely considered in nutritional recommendations of metabolic syndromes [18,19]. Therefore, stachyose application as food ingredients has increased rapidly. Soy isoflavones are mainly contain daidzin, glycitin, genistin, daidzein, glycitein and genistein, which are reported to have antioxidant, anti-inflammatory, hepatoprotective, hypoglycemic and hypolipidemic properties, as well as prevention of breast cancer [20]. However, previous studies have indicated that the bioavailability of isoflavones is extremely poor due to their poor water-solubility as well as intestinal permeability [21]. It is also well known that isoflavones easily undergo first-pass metabolism catalyzed by intestinal phase II metabolic enzymes, leading to low bioavailability of isoflavones. In a recent study, we for the first time specified that non-digestive saccharides, such as soluble soybean polysaccharides and stachyose, improved the bioavailability of soybean genistein through inhibited the expression of intestinal phase II enzymes and efflux transporters [22,23]. Nevertheless, our previous study didn't establish the mode of interaction between soybean stachyose and isoflavones in promoting body health. Meanwhile, most published studies focus on pure compounds or isolated fractions from foods for their health promoting effects without considering potential interaction among different components with the foods. In this regard, it is necessary to further investigate whether stachyose might thereby reinforce the protective effects of isoflavones against HFD-induced obesity, hyperlipidemia and hyperglycemia via enhancing the bioavailability of soybean isoflavones.

Therefore, the objective of the present study is to provide insights into the interaction between soybean stachyose and isoflavones in prevention of metabolic syndrome in C57/BL6 mice. UPLC-q/TOP-MS was employed to quantify the levels of isoflavones in the urine and feces of the tested mice. The preventive effect of stachyose or isoflavones or in combination against metabolic syndromes was investigated with the purpose to confirm whether stachyose could enhance the effect of isoflavones in HFD feeding mice. This study will serve as an indispensable basis for the development of effective whole food-based strategies for prevention of metabolic syndromes by utilizing soybean.

2. Materials and methods

2.1. Materials and Chemicals

The stachyose (pure>80%) was extracted from soybean residue [22]. Soybean isoflavones (pure>95%) includes 50% of daidzin, 6% of daidzein, 26% of glycitin, 3% of glycitein, 10% of genistin and 2% of genistein for animal experiments and the analytical standards of daidzein, glycitein and genistein (pure>98%) were bought from Shanghai Yuanye Biotechnology co., Ltd. (Shanghai, China). Assay kits of total cholesterol (TC), total triglyceride (TG), high-densitylipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), glutamic-oxal(o)acetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were purchased from HuiliBiotechnology Co. Ltd. (Changchun, China). Additionally, enzyme linked immunosorbent assay ELISA kits of interleukin-1 (IL-1), tumor necrosis factor (TNF-α), nuclear factor kappa-B (NF-κB), acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS), glucose, insulin, leptin and lipopolysaccharide (LPS) were purchased from Jiancheng Bioengineering Institute (Nanjing, China). All other reagents and chemicals were analytical grade or higher.

2.2. Animals and experiment design

Male C57/BL6 mice (weight 18-22 g) were obtained from the Experimental Animal Center of the Fourth Military Medical University. They were housed at constant temperature (22 ± 2°C) in a 12-h light/dark cycle with a minimum relative humidity of 55% - 65%. They were freely received tap water and a standard rodent chow which was also got from the Experimental Animal Center of the Fourth Military Medical University. A week after acclimatization to the laboratory environment, the mice were randomly divided into five groups (n = 8), and all of them were allowed to drink water freely during the experiment. Group ND: a normal diet group, in which the mice were offered a standard diet. Group HFD: the mice freely

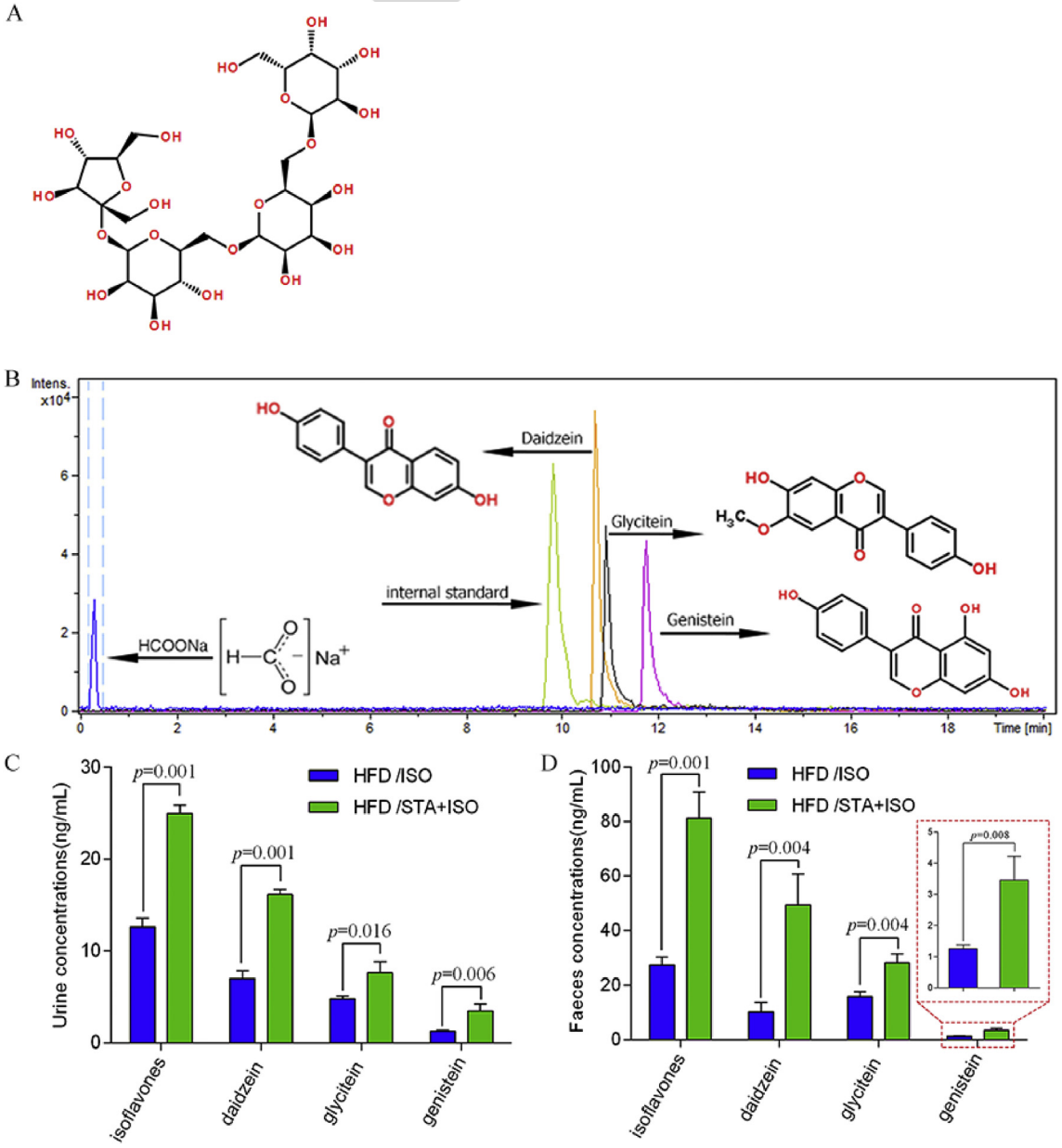

Fig. 1. The chemical structure of stachyose (A). UPLC-qTOF/MS chromatograms of a standard substance mixture and the chemical structure of daidzein, glycitein and genistein (B), and the urinary and fecal concentrations of total isoflavones, daidzein, glycitein and genistein of mice supplemented with isoflavones in HFD (HFD/ISO) and co-supplemented with stachyose and isoflavones in HFD (HFD/STA + ISO), respectively (C, D). Mean values are presented as means ± SD for 8 mice. Differences were analyzed using the Least Significant Difference (LSD) test and the differences were considered statistically at $p < 0.05$.

received high fat diet (HFD). Group HFD/STA: the mice received HFD feeds supplemented with 3% stachyose. Group HFD/ISO: the mice received HFD feeds supplemented with 1% isoflavones. Group HFD/STA + ISO: the mice received HFD feeds supplemented with 3% stachyose and 1% isoflavones together. The body weight of each mouse was measured once a week, and the consumption quantity of the food and water was monitored daily. At the end of 12 weeks, prior to the overnight fasting, all the mice were placed in metabolism cages for collecting urine and fecal samples each group. The urine and fecal samples were then stored at −80°C for further analysis. Subsequently, all the mice were anesthetized with isoflurane and then sacrificed to obtain blood, abdominal fat (epididymal + retroperitoneal + mesenteric fat) and livers. All the experiments were performed according to the Guidelines of

experimental Animal Administration published by the States Committee of Science and Technology of People's Republic of China.

2.3. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

After 12 h of fasting deprivation, the oral glucose solution (2 g/kg body weight) or an intraperitoneal injection of insulin (0.625 unit/kg body weight) was given to the animals. Whole blood glucose levels were measured by tail clipping at 0, 15, 30, 60, 90, and 120 min after glucose and insulin stimulation in all animals, respectively.

2.4. Determination of isoflavones in urine and feces by UPLC-qTOF-MS

UPLC-qTOF/MS was applied to the determination of the concentration of isoflavones in the urine and feces of mice according to previous method with proper modification [10,11]. In short, the urine of mice was centrifuged and the supernatant (200 μL) was added to 1.5 mL centrifuge tube with methanol (1000 μL). For the fecal samples, 1.5 g feces were homogenized with 9 mL normal saline, and centrifugated at 3000*g* for 15 min. The supernatant of feces (100 μL) was added to 1000 μL methanol. Finally, each sample was centrifuged and filtered with a 0.22 μm PVDF membrane to sample bottles for UPLC-qTOF/MS analysis. UPLC-qTOF/MS analysis was carried out in scan mode from 50 to 1000 (m/z) or in selected ion monitoring mode of negative (m/z) 253.2 for daidzein, (m/z) 268.9 for genistein, and (m/z) 283.2 for glycitein. And other instrument parameters are set in accordance with the previous method [11].

2.5. Measurement of serum and liver biochemical parameters

The blood samples were centrifuged at 3000*g* for 15 min to collect the serum, which was used to assess TC, TG, LDL, HDL, glucose, insulin, leptin and LPS levels, and enzymatic activities of GOT and GPT. Liver tissue (0.5 g) was homogenized with nine-fold (w/v) ice-cold normal saline and centrifuged at 1500*g* for 10 min. The supernatant was collected for the analysis of FAS, ACC, IL-1, IL-6 TNF-α and NF-κB levels. All the detailed steps of these biochemical assessments were performed according to the instructions of corresponding commercial kits.

2.6. Histopathological studies in liver and adipose tissue

Histology of liver was observed using hematoxylin and eosin (H&E) and Oil Red O staining. Livers were rapidly removed, and a little part of liver tissues were fixed by immersing in a 10% neutral formalin solution at room temperature for histological analysis. The paraffin embedded tissues were sectioned and stained with H&E. For Oil Red O staining, frozen liver samples were fixed and stained after being treated with cryostat (CM1950, Leica, Germany). Similarly, the right amount of fresh adipose tissue was cut out, fixed by immersing in a 10% neutral formalin solution at room temperature, embedded in paraffin, and stained with H&E. Finally, an Olympus light microscope was used for observation and photograph. For the analysis, the particle size distribution of fat H&E slices were measured by Nano Measurer 1.2 software, and the fat stained area of liver oil red slice were calculated by the Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA) after pictures were recorded by light phase-contrast microscopy.

2.7. Statistical analysis

All experiments have been done in triplicate and experimental data were presented as means ± SD for 8 mice each group. Datas were analyzed by one-way analysis of variance (ANOVA) and differences were analyzed using the Least Significant Difference (LSD) test. The area under the curve (AUC) for glucose levels in OGTT and ITT were calculated using the GraphPad Prism Software (v8.0.2.263). The p-values< 0.05 was considered as statistical significance. All the graphs were made using the GraphPad Prism Software (v8.0.2.263).

3. Results and discussion

3.1. Stachyose enhanced absorption of soybean isoflavones in mice

To evaluate the effects of stachyose on the absorption of isoflavones, the urinary and fecal concentrations of soybean isoflavones, including genistein, daidzein and glycitein, were identified and quantified by UPLC-qTOF-MS, and MRM chromatogram of standards mixture was also obtained by UPLC-qTOF-MS with negative ion mode (Fig. 1B). As shown in Fig. 1C, when isoflavones was supplemented together with stachyose, urine content of total isoflavones calculated as the sum of

genistein, daidzein and glycitein was significantly higher than when isoflavones supplemented alone ($p < 0.01$). It was also found that urinary concentrations of genistein, daidzein and glycitein in mice were markedly increased by the co-supplementation with stachyose and isoflavones in comparison with consumption of isoflavones alone, respectively ($p < 0.05$). Similarly, the fecal concentrations of total isoflavones, genistein, daidzein, and glyciteinin were also significantly elevated in mice co-supplementation with stachyose and isoflavones (Fig. 1D, $p < 0.01$) as compared to single supplementation of isoflavones.

Previous studies have indicated that non-digestible oligosaccharides may improve the absorption of flavonoids due to its capacity to strongly increase the stability of flavonoids in the gut [22,24]. As depicted in Fig. 1, the elevation of the fecal isoflavones levels in the mice fed with both stachyose and isoflavones indicated that stachyose might inhibit degradation of isoflavones in the gut, which was consistent with previous studies [22,24]. In this regard, we speculated that stachyose might inhibit degradation of isoflavones in the gut, and thus the bioavailability of soybean isoflavones was elevated in mice. However, understanding the exactly mechanism of the stachyose promoting absorption of isoflavones is an attractive challenge and yet requires further investigation.

3.2. Effects of co-supplementation of stachyose and isoflavones on body and fat weights

It is well known that chronically consumption of HFD can contribute to the well-established obesity, and the characteristics of obesity is of abnormal body weight, fat and blood lipid metabolism [25,26]. As shown in Table 1, there was no remarkable difference in food and water intake in all the HFD-fed mice (HFD, HFD/STA, HFD/ISO and HFD/STA + ISO, $p > 0.05$), but food and water intake of ND-fed mice significantly increased as compared to that of HFD-fed mice ($p < 0.05$), which was in accordance with the previous reports [7,10]. The tested mice among all five groups showed no significant differences in initial body weight. As expected, the HFD-fed mice gained significantly more weight than ND-fed mice on the day of necropsy ($p < 0.05$). Interestingly, the gain in body weight was decreased after treatment of mice with stachyose, isoflavones or in combination, relative to that of HFD control feeding, respectively (Table 1, $p < 0.05$). This finding strongly suggests that the decreased body and fat weights in all HF-fed mice is not due to the reduced food intake, and administrations of stachyose or/and isoflavones is one of the main reasons for the decrease of body weight and fat. Furthermore, co-consumption of both stachyose and isoflavones in mice could maximumly reduce the fat weight and fat index when compared to that of individual stachyose- or isoflavones-fed mice, respectively (Table 1, $p > 0.05$, $p < 0.05$). These results indicated that stachyose had the capacity of enhancing the efficacy of isoflavones in controlling body weight gain through suppressing the accumulation of fat.

3.3. Effects of combined consumption of stachyose and isoflavones on lipid metabolism

Studies have reported that abnormal lipid accumulation lead to metabolic disorders in mice, which is a key factor of hyperlipidemia in the progress of obesity [11]. It is well known that the HFD-induced hyperlipidemia is not only associated with the increased serum TC, TG and LDL-C levels, but is also relevant to the decrease of HDL-C levels [23]. As shown in Table 2, consumption of HFD in mice caused a severe increase in the serum TC, TG and LDL levels from 2.43, 5.79 and 1.76 mmol/L of ND-fed mice to 2.82, 7.17 and 2.77 mmol/L, respectively ($p < 0.05$), indicating that obvious lipid metabolism disorder of HFD-fed mice for 12 weeks occurred. Interestingly, the decreased trend of serum TC, TG and LDL levels was observed for all the mice

Table 1. Food intake, water intake, body weight, fat weight and fat index at the end of 12th week.

	ND	HFD	HFD/STA	HFD/ISO	HFD/STA + ISO
Food intake (g/d)	4.40 ± 0.39a	2.97 ± 0.33 b	3.02 ± 0.34 b	3.39 ± 0.48 b	3.22 ± 0.34 b
Water intake (mL/d)	3.34 ± 0.22a	2.29 ± 0.94 b	2.45 ± 1.03 b	2.34 ± 0.96 b	2.42 ± 1.03 b
Initial body weight(g)	20.63 ± 0.75a	20.97 ± 0.63a	20.59 ± 0.58a	20.54 ± 0.66a	20.36 ± 0.69a
Final body weight(g)	27.25 ± 1.14 d	32.93 ± 1.84a	31.69 ± 1.35ab	30.64 ± 0.82 b	29.19 ± 1.49c
Body weight gain(g)	6.62 ± 1.28c	11.96 ± 1.86 a	11.10 ± 1.50 ab	10.10 ± 0.96 b	8.83 ± 1.86 b
Abdominal fat weight (g)	0.59 ± 0.15c	2.29 ± 0.41a	1.89 ± 0.28ab	1.53 ± 0.39 b	1.28 ± 0.22 b
Fat index (%)	2.16 ± 0.52 d	7.01 ± 1.36a	5.97 ± 0.79ab	5.01 ± 1.34 b	4.36 ± 0.57c

ND, normal diet group; HFD, high fat diet group; HFD/STA, stachyose group; HFD/ISO, soy isoflavones group; HFD/STA + ISO, combined stachyose and isoflavones group. Fat index = abdominal fat weight/body weight × 100%; Mean values are expressed as mean ± SD for 8 mice. [a-d] Values having different significantly differences among all the groups, $p < 0.05$.

Table 2. The effects of STA, ISO or STA + ISO on serum TC, TG, HDL, LDL, and on hepatic FAS and ACC in HFD-fed mice.

	ND	HFD	HFD/STA	HFD/ISO	HFD/STA + ISO
TC (mmol/L)	2.43 ± 0.13 b	2.82 ± 0.17a	2.75 ± 0.16a	2.71 ± 0.12a	2.45 ± 0.12 b
TG (mmol/L)	5.79 ± 0.89bc	7.17 ± 0.77a	6.21 ± 0.48 b	5.49 ± 0.71c	4.08 ± 0.64 d
HDL (mmol/L)	0.89 ± 0.08a	0.93 ± 0.08a	0.88 ± 0.07a	0.89 ± 0.08a	0.86 ± 0.04a
LDL (mmol/L)	1.76 ± 0.25c	2.77 ± 0.20a	2.74 ± 0.16a	2.61 ± 0.20a	2.27 ± 0.11 b
FAS (mmol/L)	9.90 ± 1.54 d	14.6 ± 1.45a	12.8 ± 1.09 b	11.5 ± 1.45bc	10.6 ± 1.35 cd
ACC (mmol/L)	24.6 ± 3.28 b	29.3 ± 4.28a	28.5 ± 4.19ab	28.2 ± 2.02ab	24.6 ± 3.98 b

ND, normal diet group; HFD, high fat diet group; HFD/STA, stachyose group; HFD/ISO, soy isoflavones group; HFD/STA + ISO, combined stachyose and isoflavones group. Mean values are presented as means ± SD for 8 mice. [a-d] Means with different letters are significantly different, $p < 0.05$.

supplemented with different preventive diets (HFD/STA, HFD/ISO and HFD/STA + ISO), and the more significant changes in TC, TG and LDL levels were observed in the mice fed with both stachyose and isoflavones when compared to HFD control, respectively (Table 2, $p < 0.05$). HFD is well known to enhance lipid synthesis in the liver by up-regulation of FAS and ACC expression [27,28]. The present results showed that HFD markedly increased the expressions of FAS and ACC by 47.5% and 19.1% in the liver, respectively, as compared to the ND-fed mice. Furthermore, the supplementation of stachyose, isoflavones, or stachyose + isoflavones in HFD effectively protected against the increases in hepatic FAS expression levels in HFD-fed mice (Table 2, $p < 0.05$). These data indicated that the co-supplementation of stachyose and isoflavones was an effective strategy to inhibit lipid accumulation, and regulate lipid metabolism-related enzymes in HFD-fed obesity mice, as compared to single supplementation of stachyose or isoflavones.

3.4. Stachyose enhanced the effect of isoflavones in relieving liver inflammation

The HFD-induced obesity has been previously reported to cause metabolic endotoxemia, and low-grade inflammation is associated with increased intestinal permeability, as revealed by elevated circulating LPS levels [15,29]. In accordance with previous studies, long-term HFD feeding induced a significant increase in the serum LPS level by 12.7%, relative to ND-fed mice ($p < 0.05$). Although LPS levels were decreased by supplementing stachyose or isoflavones alone, there was no statistical difference, relative to HFD-fed mice. Fortunately, the combined ingestion of both stachyose and isoflavones caused the significant decrease in the serum LPS secretion in mice when compared to that of HFD-fed mice ($p < 0.05$, Fig. 2A).

To further evaluate the effects of stachyose, isoflavones or stachyose + isoflavones on hepatic inflammation, we measured the levels of IL-1, TNF-α and NF-κB in the liver. HFD dramatically elevated hepatic IL-1, TNF-α and NF-κB levels, relative to that in ND-fed mice, respectively (Fig. 2B–D, $p < 0.05$), indicating that HFD caused inflammatory responses in the liver. However, individual supplementation of stachyose or isoflavones slightly prevented up-regulation of IL-1 and TNF-α levels. Importantly, when stachyose + isoflavones was supplemented in mice, the IL-1 and TNF-α levels were significantly down-regulated by 8.18% and 9.88% as compared to that of HFD-fed mice, respectively (Fig. 2B–C, $p < 0.05$). It is well known that NF-κB as a classical signaling pathway can regulate the body's chronic inflammatory responses [30]. As seen in Fig. 2D, when the HFD-fed mice were supplemented with stachyose alone, the hepatic NF-κB levels were slightly decreased, but the reduction was not significant. However, a significant change was observed in the mice supplemented with isoflavones or stachyose + isoflavones, showing the clear reduction in the NF-κB levels by 6.13% and 9.34% relative to that in the only HFD feeding control, respectively (Fig. 2C, $p < 0.05$). This result is consistent with previous studies indicating that polyphenols, such as anthocyanin and green tea polyphenols, can target activating NF-κB pathway to inhibit the release of inflammatory factors [30-32]. Taken together, these results suggested that the combined supplementation of stachyose + isoflavones was more effective than individual treatment of stachyose or isoflavones in preventing the hepatic inflammation in HFD-induced obese mice.

3.5. Effects of stachyose and isoflavone on hyperglycemia and insulin resistance

As shown in Fig. 2E–G, continuous 12 weeks ingestion of HFD in mice prominently led to an increase in the serum fasting glucose, insulin and leptin levels by 58.60%, 30.0% and 9.08%, respectively ($p < 0.05$), suggesting that the significant hyperglycemia had happened in HFD-fed mice [8]. However, the decreased trend of serum glucose,

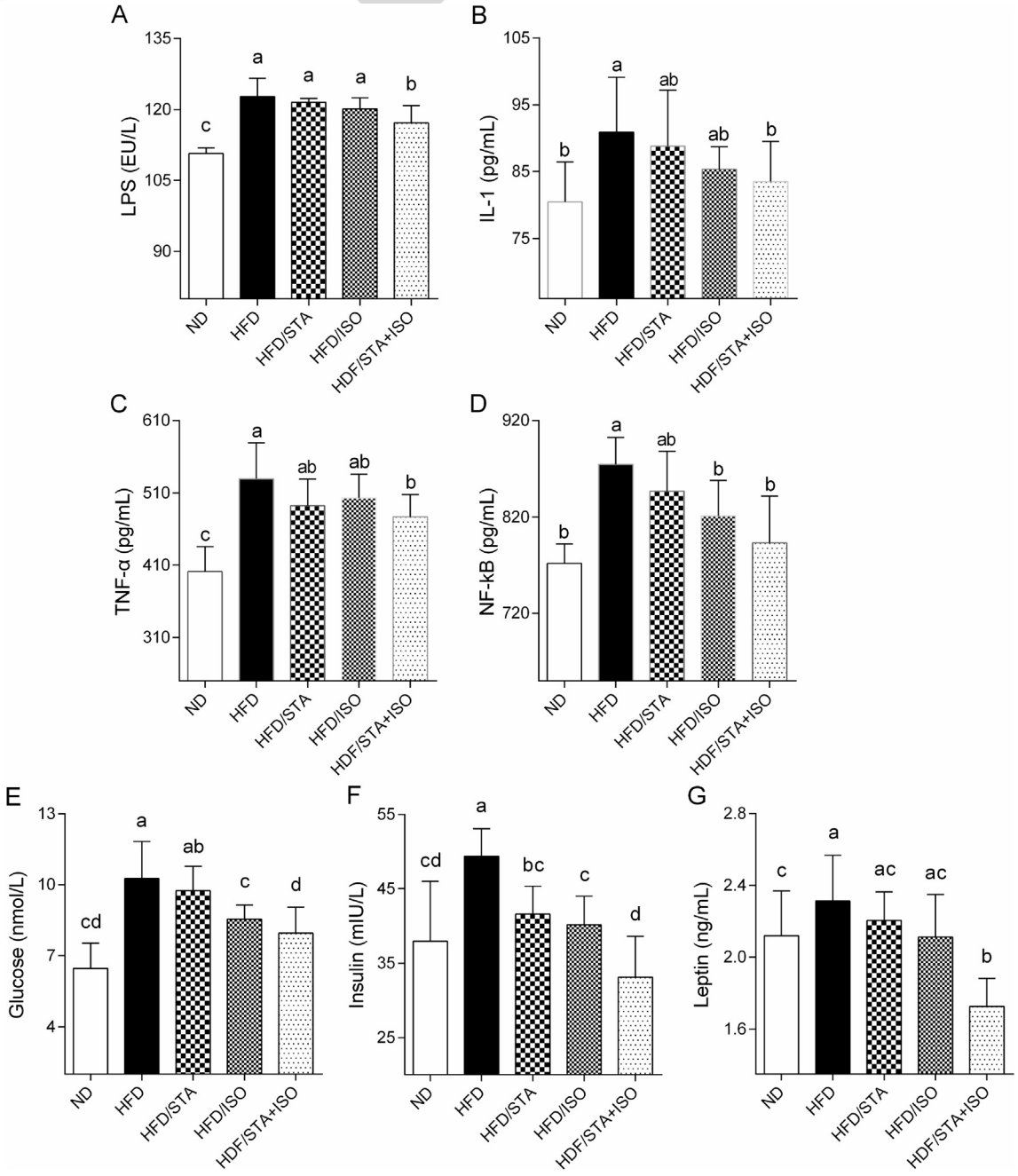

Fig. 2. Effects of stachyose, isoflavones or in combination on prevention of hepatic inflammation and hyperglycemia in HFD-fed mice. LPS (A), IL-1 (B), TNF-α (C), NF-κB (D), glucose (E), insulin (F) and leptin (G). The levels of each indicator are expressed as means ± SD (n = 8). $^{a-d}$Alphabetical letters denote significant differences ($p < 0.05$) among all groups. ND, normal diet group; HFD, high fat diet group; HFD/STA, stachyose group; HFD/ISO, soy isoflavones group; HFD/STA + ISO, combined stachyose and isoflavones group.

insulin and leptin was observed for all the protective treatments, whereas stachyose + isoflavones treatment showed more significant reduction in these parameters than individual treatment of stachyose or isoflavones (Fig. 2E–G). Furthermore, after an orally administration of glucose, the blood glucose concentration was obviously reduced by 47.4% at 30 min in mice supplemented with stachyose + isoflavones, compared to HFD-fed mice (Fig. 3A, $p < 0.05$). In addition, the AUC for glucose levels of OGTT in the mice treated with stachyose, isoflavones and stachyose + isoflavones was significantly smaller than that in HFD control mice, respectively ($p < 0.05$, Fig. 3B). Overtly, a combination of stachyose and isoflavones exhibited a prominent decrease in blood glucose levels at 15

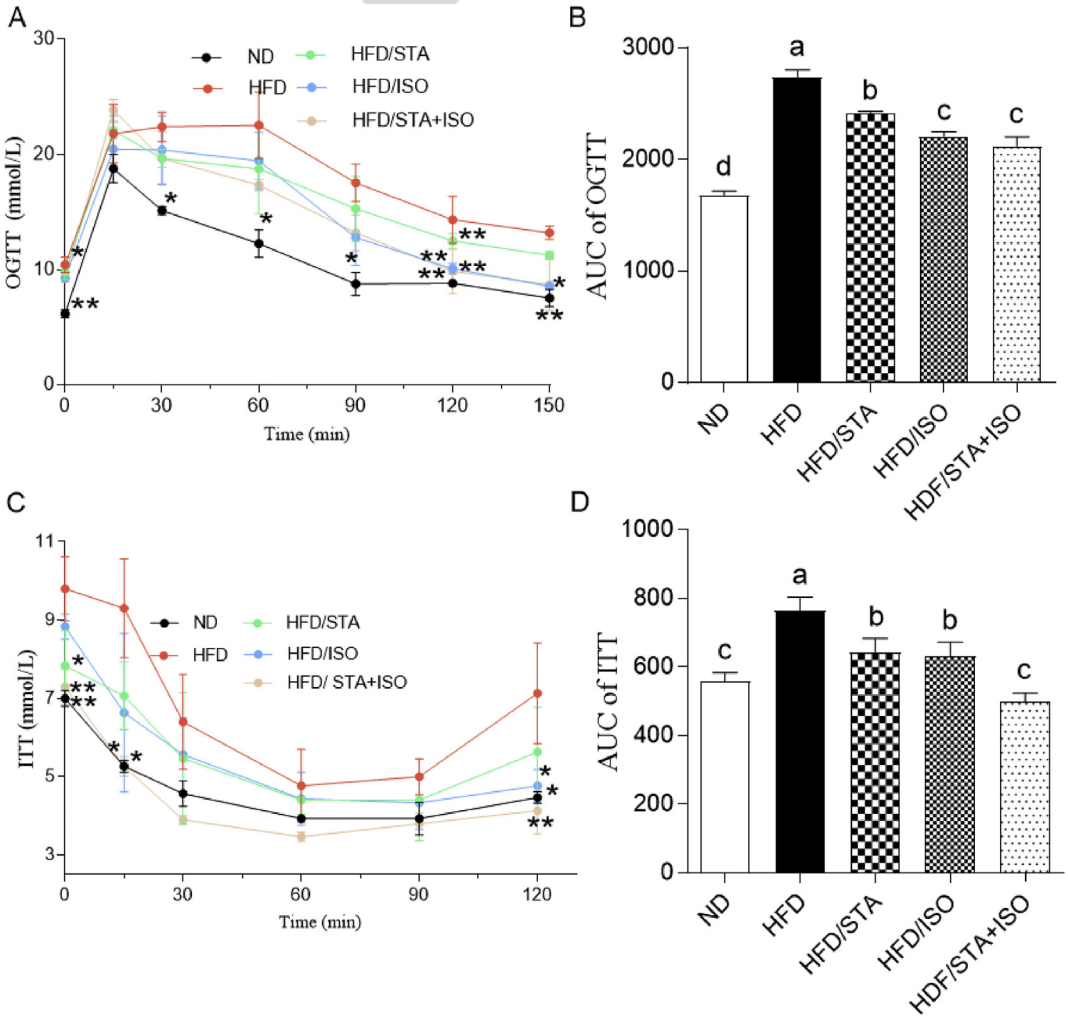

*Fig. 3. Effects of the supplementation of stachyose, isoflavones or in combination on glucose tolerance and insulin resistance in HFD-fed mice: OGTT (A), AUC of OGTT (B), ITT (C) and AUC of ITT (D). Data are expressed as means ± SD for 8 mice in each group. *$p < 0.05$ and **$p < 0.01$ vs. the HFD-fed mice in figure A and C, $^{a-d}$Alphabetical letters in figure B and D denote significant differences ($p < 0.05$) among all groups.*

min, relative to HFD control, stachyose or isoflavones feeding mice, respectively ($p < 0.05$, Fig. 3A), indicating that this combination could effectively suppress the glucose peak value than stachyose or isoflavones alone. As shown in Fig. 3C, the result of intraperitoneal ITT demonstrated that stachyose ingestion did not cause significant difference in the AUC for glucose levels, but feeding of isoflavones or stachyose + isoflavones significantly reduced the AUC in comparison with HFD control feeding, respectively ($p < 0.05$, Fig. 3D).

It is widely recognized that a persistent inflammatory response can promote glucose synthesis in the liver of mice, leading to elevated fasting glucose and insulin levels [33]. Leptin plays an important role in maintaining normal energy metabolism balance [18,34]. Normally, insulin stimulates the secretion of leptin, while the pancreas inhibits insulin secretion by binding to receptors on insulin cells [35,36]. With regard to the interaction between stachyose and isoflavones, our finding demonstrated that a combination of the two nutrients could preferably regulate the imbalance of leptin metabolism caused by HFD intake in mice (Fig. 2). Conformably, the oral glucose tolerance test and intraperitoneal insulin tolerance test indicated that stachyose enhanced the regulatory effects of isoflavones on the glucose and insulin resistance in HFD-fed mice (Fig. 3). These results demonstrated that co-supplementation of stachyose and isoflavones was more effective to regulate the glucose levels and insulin resistance than consumption of stachyose or isoflavones separately.

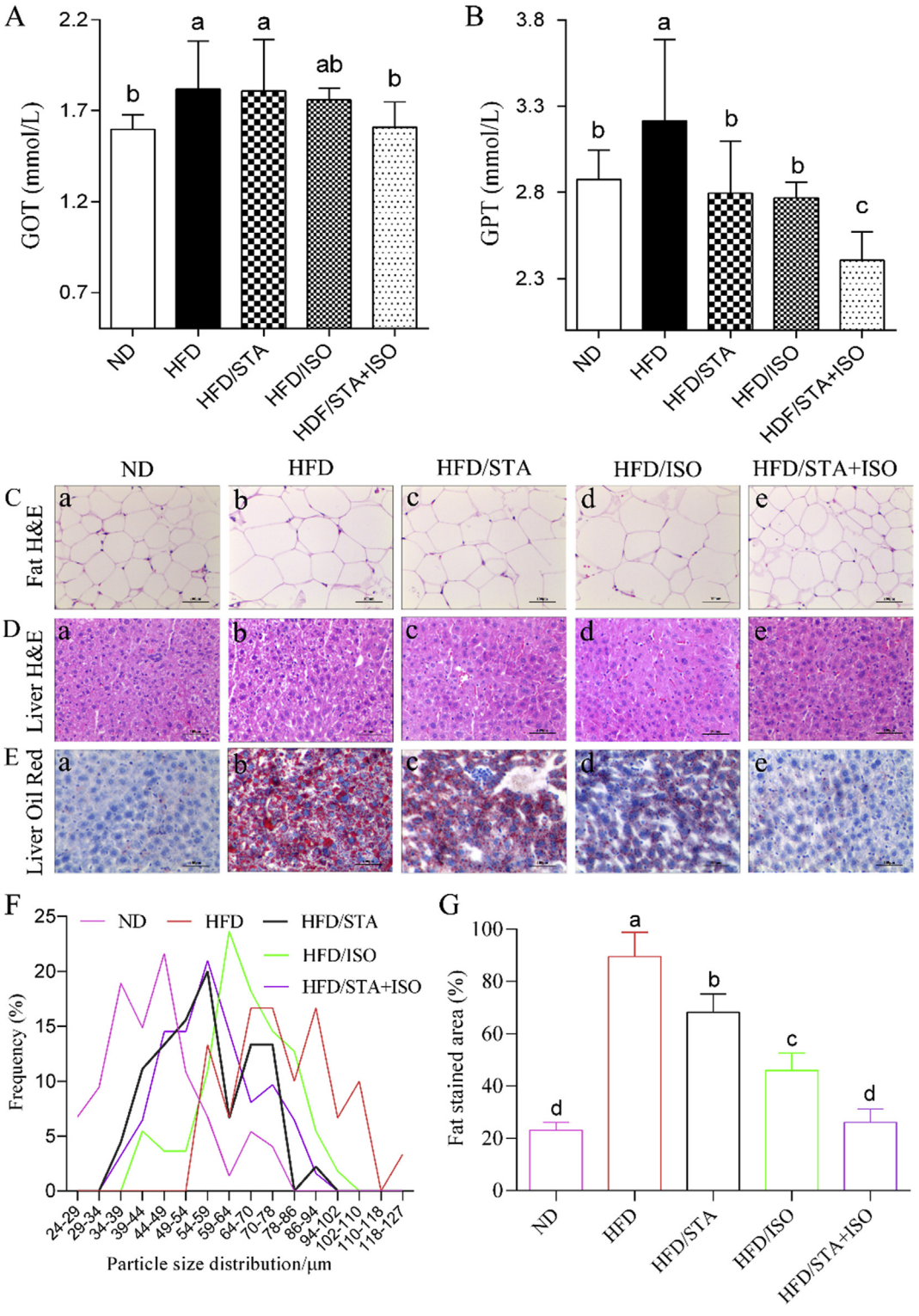

Fig. 4. Effects of the supplementation of stachyose, isoflavones or in combination against HFD-induced hepatic injury in mice: (A) Serum GOT, (B) Serum GPT, (C) Adipose tissue H&E staining, (D) liver H&E staining, (E) liver Oil Red O staining, (F) particle size distribution of fat H&E slice, and (G) the fat stained area of liver oil red slice. Results are expressed as the mean ± SD ($n = 8$). $^{a-d}$Alphabetical letters in figure A, B and G denote significant differences ($p < 0.05$) among all groups.

3.6. Stachyose improved the protection of isoflavones against hepatic injury in mice

It is well known that inflammatory response and insulin resistance are highly correlated with the formation of liver injury [9,13,36], and serum enzyme of GOT and GPT are considered as effective biochemical markers to reflect the liver functional condition [4,27]. Elevated GPT activity is a sign of cell membrane damage and the increased GOT activity is another indicator of mitochondrial damage [4,27]. In our work, the serum GOT and GPT activities of HFD-treated mice were dramatically boosted by 13.8% and 4.81% in comparison with ND-fed mice, respectively (Fig. 4A–B, $p < 0.05$). The serum GOT activity was slightly inhibited in the mice treated with stachyose or isoflavones than HFD-fed mice. Importantly, it was found that when simultaneous intake of stachyose and isoflavones in HFD-fed mice, the serum GOT activities manifested prominent inhibition ($p < 0.05$, Fig. 4B). As expected, the significant reduced trend of serum GPT activities was observed for all the protective treatments ($p < 0.05$), and more marked inhibitory effect of GPT activities were observed in the mixed stachyose and isoflavones feeding mice ($p < 0.01$, Fig. 4A).

3.7. Histopathological observations of mouse epididymis fat and liver damage

As shown in Fig. 4C/a and Fig. 4F, the adipocyte in adipose tissue of ND-fed mice showed a small diameter. However, when mice were fed with HFD, a marked increase in adipocyte size was observed by H&E staining in the adipose tissue, relative to that of ND-fed mice (Fig. 4C/b and Fig. 4F). Interestingly, a combination of stachyose together with isoflavones observably reduced adipocyte size than the supplementation of individual stachyose or isoflavones (Fig. 4C/c–f and Fig. 4F).

Fig. 4D shows photomicrographs of H&E-stained liver specimens, and the liver slices of HFD-fed mice showed the cell gap grew bigger and the loss of cellular boundaries. However, protective treatments of stachyose or isoflavones alone remitted the hepatic lesions caused by HFD (Fig. 4D/c-d). Interestingly, the liver of mice treated with stachyose + isoflavones showed near normal appearance with clear cell boundaries and complete cell structure (Fig. 4D/e), demonstrating the effective protection of combined treatment. As depicted in Fig. 4E/a-b, a widespread fat accumulation inside the parenchyma was perceived through the liver sections in HFD-Fed mice cells when compared to Oil Red O staining of liver sections from ND-fed mice. However, as shown in Fig. 4E/c-d and Fig. 4G, the livers of individual stachyose- or isoflavones-treated mice significantly improved the effect of fat deposition relative to that in HFD-fed mice. It was worth noting that the situation of hepatic fat accumulation was more obvious improvement by supplementation of both stachyose and isoflavones than individual stachyose or isoflavones (Fig. 4E/e and Fig. 4G).

4. Conclusion

The present results confirmed that stachyose significantly enhanced the bioavailability of soybean isoflavones. Furthermore, we report that stachyose effectively improved the preventive effects of isoflavones against metabolic syndrome in HFD-fed mice via mitigating the disturbance of hepatic lipid metabolism, hyperinsulinemia and inflammation. These findings suggest that the combined treatment of stachyose and isoflavones is a promising strategy to alleviate or prevent hyperlipidemia, hyperglycemia and liver injury. Finally, our study would lead to new knowledge on whole soybean and establish the mode of interaction between key fractions of soybean in promoting metabolic health.

Conflict of interest

The authors declare no conflicts of interest.

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References

1 Lee JH, Baek SY, Jang EJ, Ku SK, Kim KM, Ki SH, et al. Oxyresveratrol ameliorates nonalcoholic fatty liver disease by regulating hepatic lipogenesis and fatty acid oxidation through liver kinase B1 and AMP-activated protein kinase. Chem Biol Interact 2018;289:68–74.

2 Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19: 576–85.
3 Tortosa-Caparros E, Navas-Carrillo D, Marin F, Orenes-Pinero E. Anti-inflammatory effects of omega 3 and omega 6 polyunsaturated fatty acids in cardiovascular disease and metabolic syndrome. Crit Rev Food Sci Nutr 2017;57:3421–9.
4 Pan MH, Yang G, Li S, Li MY, Tsai ML, Wu JC, et al. Combination of citrus polymethoxyflavones, green tea polyphenols, and Lychee extracts suppresses obesity and hepatic steatosis in high-fat diet induced obese mice. Mol Nutr Food Res 2017;61.
5 Zhao L, Zhang Q, Ma W, Tian F, Shen H, Zhou MA. Combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. Food Funct 2017;8:4644–56.
6 Carey AN, Galli RL. Mitigating the effects of high fat diet on the brain and behavior with berry supplementation. Food Funct 2017;8:3869–78.
7 Luo T, Miranda-Garcia O, Sasaki G, Wang J, Shay NF. Genistein and daidzein decrease food intake and body weight gain in mice, and alter LXR signaling *in vivo* and in vitro. Food Funct 2018;9:6257–67.
8 Chen Z, Wang C, Pan Y, Gao X, Chen H. Hypoglycemic and hypolipidemic effects of anthocyanins extract from black soybean seed coat in high fat diet and streptozotocin-induced diabetic mice. Food Funct 2018;9:426–39.
9 Fan JG. Impact of non-alcoholic fatty liver disease on accelerated metabolic complications. J Digest Dis 2008;9:63–7.
10 Lu Y, Li W, Yang X. Soluble soybean polysaccharides enhance the protective effects of genistein against hepatic injury in high L-carnitine-fed mice. Food Funct 2017;8:4364–73.
11 Lu Y, Zhao A, Wu Y, Zhao Y, Yang X. Soybean soluble polysaccharides enhance bioavailability of genistein and its prevention against obesity and metabolic syndrome of mice with chronic high fat consumption. Food Funct 2019;10:4153–65.
12 Pan MH, Tung YC, Yang G, Li S, Ho CT. Molecular mechanisms of the anti-obesity effect of bioactive compounds in tea and coffee. Food Funct 2016;7:4481–91.
13 Chen Q, Wang T, Li J, Wang S, Qiu F, Yu H, et al. Effects of Natural Products on Fructose-Induced Nonalcoholic Fatty Liver Disease (NAFLD). Nutr 2017;9:96.
14 Liu W, Zhao S, Wang J, Shi J, Sun Y, Wang W, et al. Grape seed proanthocyanidin extract ameliorates inflammation and adiposity by modulating gut microbiota in high-fat diet mice. Mol Nutr Food Res 2017;61.
15 Lopez P, Sanchez M, Perez-Cruz C, Velazquez-Villegas LA, Syeda T, Aguilar-Lopez M, et al. Long-Term Genistein Consumption Modifies Gut Microbiota, Improving Glucose Metabolism, Metabolic Endotoxemia, and Cognitive Function in Mice Fed a High-Fat Diet. Mol Nutr Food Res 2018;62.
16 Xu Q, Chao Y, Wan Q. Health benefit application of functional oligosaccharides. Carbohyd Poly 2009;77:435–41.
17 Zhong X, Zhang Y, Huang G, Ouyang Y, Liao D, Peng J. Proteomic analysis of stachyose contribution to the growth of Lactobacillus acidophilus CICC22162. Food Funct 2018;9: 2979–88.
18 Zhao C, Wu Y, Liu X, Liu B, Cao H, Yu H, et al. Functional properties, structural studies and chemo-enzymatic synthesis of oligosaccharides. Food Sci Tech 2017;66:135–45.
19 Yang X, Zhao Y, He N, Croft KD. Isolation, Characterization and Immunological Effects of the Alpha-Galacto-oligosaccharides from a New Source, the Herb Lycopus lucidus Turcz. J Agric Food Chem 2010;58:8253–8.
20 Franke AA, Lai JF, Halm BM. Absorption, distribution, metabolism, and excretion of isoflavonoids after soy intake. Arch Biochem Biophys 2014;559:24–8.
21 Wang S-T, Fang T-F, Hsu C, Chen C-H, Lin C-J, Su N-W. Biotransformed product, genistein 7-O-phosphate, enhances the oral bioavailability of genistein. J Funct Foods 2015;13: 323–35.
22 Lu Y, Lin D, Li W, Yang X. Non-digestible stachyose promotes bioavailability of genistein through inhibiting intestinal degradation and first-pass metabolism of genistein in mice. Food Nutr Res 2017;61:1369343.
23 Lu Y, Li W, Yang X. Soybean soluble polysaccharide enhances absorption of soybean genistein in mice. Food Res Int 2018; 103:273–9.
24 Noriko M, Megumi M, Hideyuki C, Hiroshi H. Oligosaccharide Promotes Bioavailability of a Water-Soluble Flavonoid Glycoside, rG-Rutin, in Rats. J Agric Food Chem 2009;57: 1498–505.
25 Jang A, Kim D, Sung KS, Jung S, Kim HJ, Jo C. The effect of dietary alpha-lipoic acid, betaine, l-carnitine, and swimming on the obesity of mice induced by a high-fat diet. Food Funct 2014;5:1966–74.
26 Chiu CY, Wang LP, Liu SH, Chiang MT. Fish oil supplementation alleviates the altered lipid homeostasis in blood, liver, and adipose tissues in high-fat diet-fed rats. J Agric Food Chem 2018;66:4118–28.
27 Wang X, Meng Y, Zhang J. Ezetimibe alleviates non-alcoholic fatty liver disease through the miR-16 inhibiting mTOR/p70S6K1 pathway. RSC Adv 2017;7:37967–74.
28 Meriga B, Parim B, Chunduri VR, Naik RR, Nemani H, Suresh P, et al. Antiobesity potential of Piperonal: promising modulation of body composition, lipid profiles and obesogenic marker expression in HFD-induced obese rats. Nutr Metab 2017;14:72.
29 Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free Radic Biol Med 2012;52:59–69.
30 Real JT, Martinez-Hervas S, Garcia-Garcia AB, Civera M, Pallardo FV, Ascaso JF, et al. Circulating mononuclear cells nuclear factor-kappa B activity, plasma xanthine oxidase, and low grade inflammatory markers in adult patients with familial hypercholesterolaemia. Eur J Clin Invest 2010;40:89–94.
31 Khan MS, Ali T, Kim MW, Jo MH, Chung JI, Kim MO. Anthocyanins improve hippocampus-dependent memory function and prevent neurodegeneration via JNK/Akt/GSK3 signaling in LPS-treated adult mice. Mol Neurobiol 2019;56:671–87.
32 Yang F, de Villiers WJ, McClain CJ, Varilek GW. Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr 1998;128: 2334–40.
33 Sun XJ, Kim SP, Zhang D, Sun H, Cao Q, Lu X, et al. Deletion of interleukin 1 receptor-associated kinase 1 (Irak1) improves glucose tolerance primarily by increasing insulin sensitivity in skeletal muscle. J Biol Chem 2017;292:12339–50.
34 Pekmez CT, Dragsted LO, Brahe LK. Gut microbiota alterations and dietary modulation in childhood malnutrition - The role of short chain fatty acids. Clin Nutr 2019;38:615–30.
35 Mehebik-Mojaat N, Ribiere C, Niang F, Forest C, Jaubert AM. Leptin and insulin induce mutual resistance for nitric oxide synthase III activation in adipocytes. J Cell Biochem 2009;108: 982–8.
36 Li W, Li Z, Han X, Huang D, Lu Y, Yang X. Enhancing the hepatic protective effect of genistein by oral administration with stachyose in mice with chronic high fructose diet consumption. Food Funct 2016;7:2420–30.