Bioactive Constituents, Metabolites, and Functions

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Dietary Genistein Reduces Methylglyoxal and Advanced Glycation End Product Accumulation in Obese Mice Treated with High-Fat Diet

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ABSTRACT

Our previous study has found that dietary genistein could ameliorate high-fat diet (HFD)-induced obesity and especially lower methylglyoxal (MGO) and advanced glycation end products (AGEs) accumulation in healthy mice exposed to genistein and HFD. However, it is still unclear whether dietary genistein intervention has a similar beneficial effect in obese mice. In this study, the mice were induced with obesity after being fed a HFD for nine weeks before being administered with two doses of genistein, 0.1% (G 0.1) and 0.2% (G 0.2), in the HFD for additional 19 weeks, respectively. After 19-week treatment, genistein supplementation reduced body and liver weights, plasma and liver MGO levels, and kidney AGEs levels in mice. Mechanistically, genistein upregulated the expressions of glyoxalase I and II, and aldose reductase to detoxify MGO, and genistein and its microbial metabolites, dihydrogenistein and 6'-hydroxy-O-demethylangolensin, were able to trap endogenous MGO *via* formation of MGO conjugates. Taken together, our results provide novel insights into the anti-obesity and anti-glycation roles of dietary genistein in obese subjects.

KEYWORDS: genistein, methylglyoxal, advanced glycation end products, detoxification enzyme, trapping, obesity

INTRODUCTION

Methylglyoxal (MGO), a highly reactive dicarbonyl compound and potent glycating agent, is an ubiquitous metabolite that spontaneously reacts with proteins and DNA forming advanced glycation end products (AGEs). As the major precursor of AGEs, MGO can be generated endogenously as a byproduct of glycolysis and several other metabolic pathways even under physiological circumstances.[1-3] The glyoxalase system, the most important system in the metabolism of MGO, and other enzymes play an important role in regulating MGO levels.[2, 4-5] Moreover, MGO is also present in many food products, such as cookie, bread, cheese, and carbonated soft drinks, and in cigarette smoke as exogenous sources.[6-8] MGO and MGO-derived AGEs can afflict organs and tissues affecting their functions and structure. Accumulating evidence highlights that the formation and accumulation of MGO and AGEs are strongly associated with aging-related diseases, including cancer, neurodegenerative diseases, diabetes, and obesity.[2-4, 9-13] Therefore, lowering MGO levels would be an effective approach to inhibit the formation of AGEs and ameliorate the associated conditions of diseases.

Genistein (4',5,7-trihydroxyisoflavone), the principal soy isoflavone, is widely used as a dietary supplement in the United States.[14-15] Many studies have found that genistein has many biological activities, such as anti-diabetes, anti-obesity, anti-cancer, antioxidant, anti-inflammation, and inhibition of tyrosine-specific protein kinases.[14, 16-22] As a selected, effective, and safe natural bioactive compound for the prevention of diabetes and obesity, our previous studies have shown that genistein can effectively trap MGO and inhibit AGE formation *in vitro* and *in vivo*.[7, 23-24] Importantly, dietary genistein intervention alleviates the very-high fat diet (VHFD, 60% energy from fat) and HFD (45% energy from fat) plus exogenous MGO-induced metabolic syndrome (MetS) and lowers the MGO and MGO-induced AGE accumulation *via*

trapping MGO and mediating the detoxification pathways of MGO.[7] Started with young healthy mice, co-treatment with HFD, dietary genistein has shown the potential beneficial effects on lowering of MGO and AGEs accumulation in mice, suggesting genistein has the potential to prevent the MGO and AGE accumulation in healthy individuals eating fat-enriched foods. However, it is still unclear whether dietary genistein intervention has the same or similar beneficial effects in overweight and obese individuals. To mimic this situation in the current study, young healthy mice were fed a HFD for nine weeks to induce obesity before administering genistein, and then the obese mice were given two doses of dietary genistein for the following 19 weeks. The objective of this study is to elucidate the impact of genistein on the accumulations of MGO and AGEs in obese mice and its underlying mechanisms to detoxify MGO.

MATERIALS AND METHODS

Chemicals and Reagents

Methylglyoxal solution (40% in water) and other chemicals were obtained from Sigma (St. Louis, MO, USA). Genistein was purchased from LC Laboratories (Woburn, MA). The antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) and Cell Signaling (Danvers, MA, USA). HPLC-grade and LC/MS-grade solvents and other reagents were obtained from VWR International (South Plainfield, NJ, USA) and Thermo Fisher Scientific (Pittsburgh, PA, USA).

Animal treatment

Five weeks old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed according to the experimental protocol approved by the Institutional Animal Care and Use Committee of the North Carolina Research Campus (Protocol number 15-010).

After one week of acclimation, all mice were fed the high-fat diet (HFD, 45% energy from fat, D12451) and water *ad libitum* for nine weeks to induce moderate obesity with 20% - 25% greater body weight than control mice. Then, all the obese mice were randomly divided into three groups (n=10 in each group) given the following treatments for 19 weeks: (1) high-fat (HF) group, given HF diet; (2) 0.1% genistein group (G 0.1), given 0.1% (w/w) genistein in HF diet; (3) 0.2% genistein group (G 0.2), given 0.2% (w/w) genistein in HF diet. All the special diets were prepared by and ordered from Research Diets (New Brunswick, NJ). The exact nutrient composition of the diets is shown in **Table 1**.

The body weight (BW) and food intake were recorded weekly. The 24-h fecal samples were collected using a metabolic cage and then frozen at -80 °C until further analysis. The mice were sacrificed at the end of the experiment and the samples of blood, liver, kidney, and adipose tissues (epididymal (Ep), retroperitoneal (Rp), and mesenteric fat) were harvested, weighed, frozen rapidly in liquid nitrogen, and stored at -80 °C until further analysis. The outline of the study design was shown in **Fig. 1**.

Biochemical analyses of plasma and liver samples

Blood samples were collected by cardiac puncture and plasma was obtained by centrifuging the blood at 2,000 g for 15 min at 4 °C. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma and levels of cholesterol and triglyceride (TG) in plasma and liver were measured as described previously.[7, 25] Fasting blood glucose levels (6 h fasting) were measured at week 18 following the previous procedure.[25]

Measurement of MGO and AGEs levels

Plasma MGO levels were measured using the LC-MS method described previously.[7] 1, 2-diaminobenzene was employed to incubate with the supernatant of the deproteinized plasma

sample for 3 h at 37 °C in the dark to form the specific quinoxaline derivate, 2-methylquinoxaline (2-MQ). Then, 2-MQ and the internal standard 2, 3-dimethylquinoxaline (DMQ) were detected by LC-MS.

The total AGEs levels in plasma, liver, and kidney tissues were measured based on the fluorescence absorption as described previously.[7, 25]

Western blot assay

Liver and kidney tissues were prepared in RIPA buffer as whole protein lysates and the expressions of MGO detoxification related enzymes (glyoxalase I/II (GLO I/II), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), and aldose reductase (AR)) and receptor of AGEs (RAGE) were investigated by western blot as described previously.[7, 25]

Fecal sample preparation

Dried feces (~50 mg) from each group (HF, G 0.1, and G 0.2) were separately ground and soaked into 0.5 mL methanol, and subsequently homogenized for 60 s (10 cycles) by a Bead Ruptor Homogenizer (Omni International, Kennesaw, GA), and then sonicated for 30 min. The suspension was centrifuged at 16000 g for 10 min. The supernatant was removed. The process of sonication was repeated once with the addition of another 0.5 mL MeOH. The supernatants were combined and evaporated under a gentle stream of nitrogen. The residue was reconstituted into 50 μL 95% MeOH (0.1% FA). After centrifugation at 16000 g for another 10 min, the supernatant was removed for LC-MS analysis.

HPLC-MS analysis

Chromatographic separation of major MGO conjugates of genistein was achieved on a Gemini C18 column (3.0 i.d. × 150 mm, 5 μm) (Phenomenex, Torrance) using a Vanquish Ultra-High

Pressure Liquid Chromatography (UHPLC) (Thermo Scientific, San Jose). The binary mobile phase system consisted of water with 0.1% formic acid (FA) (phase A) and acetonitrile with 0.1% FA (phase B). The flow rate was 0.2 mL/min, and the injection volume was 5 μL. The column was eluted with a gradient program (0% B from 0 to 2 min, 0 to 25% B from 2 to 15 min, 25 to 88% B from 15 to 25 min, 88 to 100% B from 25 to 26 min, maintaining 100% B for 3 min and back to 0% B in 1 min, and then re-equilibrated with 0% B for another 3 min). The identification of major MGO conjugates was conducted with a Thermo Scientific Q Exactive Plus mass spectrometer system (Thermo Scientific, San Jose) equipped with a heated electrospray ionization source (HESI-II). The negative ion polarity mode was set for an ESI ion source with the voltage on the ESI interface maintained at approximately 2.50 kV. Temperatures of the HESI-II auxiliary gas heater and capillary were set as 250 °C and 300 °C. Sheath, auxiliary, and sweep gases were operated at 45, 10 and 2 AU. The S-lens RF level was set at 65. The scanning duration (from 10 to 27min) was determined under Full Mass mode with a mass range of *m/z* 100 to 1000. The resolution for Full Scan was acquired at 70,000 FWHM (full width at half maximum) with an automatic gain control (AGC) of 3×10^6 and a maximum injection time (IT) of 100 msec. The MS^1 data of the potential MGO conjugates were transferred into inclusion list with a specific NCE of 40 eV for the parallel reaction monitoring (PRM) analysis. MS^2 data in PRM mode were acquired with a resolution at 35,000, AGC target at 3×10^6, maximum IT at 120 ms, isolation window at 2.0 *m/z*, and stepped NCE at 15, 35, and 60 eV. The mass range was measured from *m/z* 50 to 800. Data were acquired in time-scheduled PRM events using the Thermo Fisher Scientific Xcalibur Quan Browser (Version 4.1.31.9).

Synthesis of authentic standards

6'-Hydroxy-O-demethylangolensin (6'-OH-DMA). 6'-OH-DMA was synthesized in house by following the reported method.[26] In detail, to a stirred slurry of lithium aluminium hydride (77 mg, 5.5 equv.) in reflux with THF (5 mL) was added to a solution of genistein (100 mg, 1.0 equv.) in THF (10 mL) over 30 min. After further refluxing for 2.5 h, the reaction mixture was cooled and poured into saturated NH_4Cl aqueous solution at 0 ^oC. The mixture was neutralized with 2 M HCl and extracted with ethyl acetate (EA). The EA layer was dried over Na_2SO_4 and filtered. The filtration was evaporated *in vacuo*. The residue was loaded onto a column silica gel column (H/E = 10:1, 5:1, 2:1, 1:1, and 1:2) and followed by repeated Pre-TLC (C/M = 8:1), giving rise to the target compound (47 mg, yield: 47%) as a light brown solid. ^{1}H-NMR (600 MHz, MeOD-*d4*) δ 5.23 (1H, q, *J* = 7.0 Hz, H-2), 1.40 (3H, d, *J* = 7.0 Hz, H-3), 5.78 (2H, s, H-3'/5'), 7.12 (2H, d, *J* = 8.5 Hz, H-2"/6"), and 6.69 (2H, d, *J* = 8.5 Hz, H-3"/5"); ^{13}C-NMR (100 MHz, MeOD-*d4*) δ 209.1 (s, C-1), 50.8 (d, C-2), 20.7 (q, C-3), 106.0 (s, C-1'), 166.6 (s, C-2'/6'), 96.7 (d, C-3'/5'), 166.8 (s, C-4'), 135.7 (s, C-1"), 131.1 (d, C-2"/6"), 116.8 (d, C-3"/5"), and 157.8 (s, C-4").

MGO conjugates of genistein (MGO-GEN) and 6'-OH-DMA (MGO-6'-OH-DMA). *In situ* synthesis of MGO-GEN and MGO-6'-OH-DMA was carried out using our previous method with some modifications.[27] In detail, a mixture of MGO (0.5 mM) and each flavonoid (genistein or 6'-OH-DMA, 1.5 mM) in PBS (100 mM, pH 7.4) was incubated at 37 ^oC for 3 h. Then, acetic acid (3 μL) was added to stop the reaction. The reaction medium was subsequently diluted 1000 times for MGO-GEN or 10,000 times for MGO-6'-OH-DMA with 95% MeOH (0.1% FA) for LC-MS analysis. The extracted ions were used as the according authentic references.

NMR Analysis

^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AVANCE 600 MHz spectrometer (Bruker, Silberstreifen, Rheinstetten, Germany). All compounds were analyzed in MeOD-*d4*. Multiplicities

155 are indicated by *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), and *br* (broad). The ^{13}C-NMR spectra
156 are proton-decoupled.

157 **Statistical analysis**

158 All values are expressed as means ± standard deviation (SD). One-way ANOVA with Tukey's test
159 was performed to analyze the difference using GraphPad Prism 8. A p-value < 0.05 was considered
160 significant.

161

162 **RESULTS**

163 **Dietary genistein inhibited HFD-induced body weight gain in obese mice**

164 After the mice were fed a HFD for nine weeks to induce obesity, the BW was increased from 21.87
165 ± 1.80 g to 36.98 ± 3.58 g, which is consistent with our previous data as a moderate obesity.[7, 28-29]
166 Dietary genistein supplementation at the dose of 0.2% significantly reduced the BW starting from
167 week 2 (by 9.7%) to week 19 (by 12.3%) compared with the mice in the HF group, and 0.1%
168 genistein treatment also decreased the BW from week 14 by 4.4% to week 19 by 3.0%, but was
169 not statistically significant compared with HF mice due to the large individual variation (**Fig. 2A**).
170 In **Fig. 2A**, the BW of the mice in the HF group at week 9 dropped down suddenly due to an
171 accident with the water bottle leaking, but the mice recovered quickly after solving the problem.
172 Also, the slight drop in BW at week 19 was due to the fasting of mice for 6 h to measure blood
173 glucose levels during week 18. Additionally, both doses of genistein slightly reduced the food
174 intake (3% for G 0.1 and 5.8% for G 0.2) compared with the HF group (**Fig. 2B**).

175 There was no change in the weights of the kidney and total fat after administering dietary
176 genistein (**Fig. 2C**). Also, the weights of Rp fat and mesenteric fat were decreased by
177 approximately 19.5% and 19.3% by 0.2% genistein treatment, respectively, compared with HF

mice, but not statistically significant due to the large individual variation. (**Fig. 2C**). Importantly, genistein at 0.2% significantly decreased the liver weight by 36.2% compared with HF mice (**Fig. 2C**, 1.85 ± 0.63 g *vs* 2.90 ± 0.42 g). However, genistein treatment at 0.2% significantly increased the weight of Ep fat (2.15 ± 0.28 g) compared with HF (1.73 ± 0.54 g) and G 0.1 (1.65 ± 0.18 g) mice (**Fig. 2C**).

Effects of dietary genistein on biochemical parameters in obese mice

After 18 weeks of dietary genistein consumption, the fasting glucose concentration was significantly decreased in the G 0.1 group (by 10%, 150.4 ± 9.08 mg/dL), but not in the G 0.2 group (166.6± 12.18 mg/dL), compared with HF mice (165.7 ± 13.82 mg/dL, **Fig. 3**). As shown in **Fig. 3**, dietary genistein intervention did not affect the activity of AST and liver TG level, but slightly decreased the ALT levels by 25% (G 0.1, 10.48 ± 4.29 U/L) and 16.7% (G 0.2, 11.64 ± 4.79 U/L) respectively, but not statistically significant compared with HF mice (13.97 ± 2.13 U/L, **Fig. 3**) due to the large individual variation. In addition, plasma TG and cholesterol levels were slightly reduced by 7.98% (21.49 ± 3.09 mg/dL) and 18.2% (114.98 ± 42.26 mg/dL) in genistein at 0.2% treatment, respectively, but not significantly compared with HF mice (TG, 29.47 ± 10.35 mg/dL; cholesterol, 140.5 ± 10.35 mg/dL, **Fig. 3**). However, plasma TG concentration was significantly lowered by 10.7% in G 0.1 group (18.78 ± 6.58 mg/dL, **Fig. 3**).

Effects of dietary genistein on MGO and AGEs levels in obese mice

Dietary genistein supplementation at 0.2% significantly decreased the MGO levels in the plasma and liver by 43.9% (122.2 ± 48.76 nM) and 30.4% (5.93 ± 1.31 nmol/g) compared with HF mice (217.92 ± 18 nM and 8.53 ± 1.89 nmol/g, respectively, **Fig. 4**). In addition, the kidney MGO level was slightly decreased by 12.5% in G 0.2 group, but not statistically significant compared with the HF group (**Fig. 4**).

Furthermore, the kidney AGE level was significantly lowered by 48.3% in dietary genistein treatment at 0.2% and decreased by 26% in G 0.1 group, but not statistically significant due to large individual variation (**Fig. 4**). However, there was no significant change of AGE levels in plasma and liver after dietary genistein treatments.

Dietary genistein activated MGO detoxification systems and blocked AGE/RAGE pathway in obese mice

The expressions of GLO I/II, GCLC, and GCLM, the important enzymes in the MGO detoxification glyoxalase system, were determined in kidney and liver samples. In the kidney, the expressions of GLO I and GLO II were significantly increased by dietary genistein treatment at 0.2% (by 1.29-fold and 1.32-fold, respectively, **Fig. 5**), but not by 0.1% genistein treatment. Similarly, the expressions of GCLC and GCLM were also elevated by genistein treatment at 0.2% (by 1.24-fold and 1.20-fold) compared with HF mice (**Fig. 5**). Importantly, the expression of AR, another MGO detoxification enzyme, in the kidney was dramatically increased over 3-fold by 0.2% genistein treatment compared with HF mice (**Fig. 5**). Moreover, dietary genistein treatment at 0.2% significantly lowered the RAGE expression by 25% in the kidney compared with HF mice (**Fig. 5**), indicating that the AGE/RAGE pathway was interrupted.

Similarly, the expressions of GLO I and GLO II in the liver were significantly elevated by 1.25- fold and 3-fold in dietary genistein treatment at 0.2%, respectively (**Fig. 6**). Meanwhile, the expressions of GCLC and GCLM were also increased by 1.27-fold and 1.32-fold in the G 0.2 group compared with HF mice (**Fig. 6**). However, dietary genistein treatments did not significantly alter the expressions of AR in the liver compared with HF mice. Moreover, the RAGE expression in the liver was decreased significantly by 40% with genistein treatment at 0.2% (**Fig. 6**).

Dietary genistein trapped endogenous MGO in obese mice

Our previous studies showed that genistein and its microbial metabolites, such as dihydrogenistein (DHGEN) and 6'-OH-DMA, are able to trap MGO *via* formation of MGO conjugates in mice.[7, 24] In this work, MGO-GEN and MGO-6'-OH-DMA were synthesized *in situ* and used as references. The excretion of MGO-GEN (*m/z* 341.0667 $[M-H]^-$) was characterized by the predominant MS^2 ion at *m/z* 269.0458 $[M-MGO-H]^-$ (**Fig. 7A**). Typical MS^2 fragments at *m/z* 327.0879 $[M-H_2O-H]^-$, 312.0644 $[M-H_2O-Me-H]^-$, 299.0929 $[M-H_2O-CO-H]^-$, and 273.0771 $[M-MGO-H]^-$ confirmed the identity of MGO-6'-OH-DMA (**Fig. 7B**). In addition, the daughter ion at *m/z* 165.0196 $[M-MGO-C_7H_6O-H]^-$, corresponding to the formation of 4,6-dihydrooxybenzofuran-3-one and as evidence for the DHGEN moiety,[30] suggested the presence of MGO-DHGEN (**Fig. 7C**). As shown in **Fig. 7**, three MGO conjugates, including MGO-GEN, MGO-6'-OH-DMA, and MGO-DHGEN, appeared in mouse feces after genistein intake in a dose-dependent manner, but not in HF group.

DISCUSSION

The current study demonstrated for the first time that dietary genistein at 0.2% significantly decreased the accumulations of MGO and AGEs in obese mice. Further mechanistic studies showed that dietary genistein reduced the accumulations of MGO and AGEs *via* two different pathways, activation of MGO detoxification pathways and directly trapping MGO to form the corresponding MGO conjugates. The similar beneficial effects of dietary genistein (0.25% and 0.2% in the HFD, respectively) on inhibiting MGO and AGEs accumulations have also been found in our previous preventive study in either VHF (VHF, 60 kcal% fat) or HF (45 kcal% fat) plus exogenous MGO-induced MetS in young healthy mice. [7] Therefore, it suggests that genistein may

246 be a candidate agent to prevent unhealthy food-induced MetS and MGO-AGEs associated chronic
247 diseases in both healthy and obese individuals.

248 It has been reported that overexpression of GLO I, the major MGO detoxification enzyme, and
249 GLO I inducer could reduce the MGO level and AGE formation *in vivo*.[31-32] Even though genistein
250 has not been reported as a GLO I inducer, it was found to have the capacity to upregulate the
251 expressions of GLO I and II, [7] and normalize the high-fructose diet-induced reductions of GLO I
252 and II activity in rats.[33] In this study, both GLO I and GLO II were significantly upregulated by
253 0.2% genistein treatment in the liver and kidney of obese mice, which are consistent with the
254 findings in our previous genistein studies.[7] Also, the two GSH synthase enzymes (GCLC/GCLM)
255 were upregulated by 0.2% genistein treatment in obese mice, indicating that the activation of the
256 glyoxalase system may lower the concentrations of endogenous MGO. Moreover, our results
257 demonstrated that the expression of AR, another important enzyme for MGO detoxification, was
258 significantly upregulated by 0.2% genistein treatment in the kidney, which is consistent with our
259 previous genistein study started with young healthy mice [7]. However, the AR expression in the
260 liver was not altered by genistein in obese mice, which is different from our previous genistein
261 studies started with young healthy mice [7]. Therefore, dietary genistein has the capacity to decrease
262 MGO accumulation *via* activating the glyoxalase system and upregulating the AR expression in
263 HFD-treated mice that were initially either healthy or obese. Similar to our previous experiments,
264 the significantly down-regulated RAGE expressions in the kidney and liver indicated that the
265 AGE/RAGE pathway was interrupted by dietary genistein at 0.2%.

266 In this study, besides the mono-MGO conjugate of genistein observed in our previous studies,
267 the mono-MGO adducts of the microbial metabolites of genistein, such as DHGEN and 6'-OH-
268 DMA, were also found in genistein treated mice, indicating that the MGO trapping mechanism

plays a role in lowering MGO levels in both healthy and obsese mice and gut microbiota may impact the *in vivo* trapping efficacy of genistein. Taken together, genistein can inhibit HFD induced MGO and AGEs accumulations through both trapping of MGO (direct effect) and activation of MGO detoxification systems (indirect effect).

The beneficial effects of genistein against obesity and diabetes associated MetS have been reported in numerous studies.[16, 34-44] In most of these studies, young healthy mice were used to start the co-treatment of HFD and genistein.[7, 40-42, 44-46] There is only one very recent study that began testing the effect of genistein against HFD-induced MetS after the mice were induced with obesity. In that study, a single dose of genistein (0.02% in HFD (40% fat)) was administered to the obese female ICR mice and the HFD induced BW gain was significantly decreased after 4 weeks of treatment,[47] which is contradictory to the results of genistein at 0.1% started with obese mice in the current study and genistein treatment at 0.067% in healthy mice that were fed a HFD plus MGO in our previous study.[7] In the current and previous studies, we only observed that high doses of genistein (0.2% and 0.25%) significantly decreased HFD-induced BW gain. This may due to different gender (male *vs* female) and strain (C57 BL/6J *vs* ICR) of mice that were used in our studies *vs* the recently reported study.

In this study, we observed that the inhibitory effects of genistein on HFD-induced BW gain and elevated biomarkers related to MetS in obese mice were not as strong as those in the young healthy mice given the same treatment from the beginning. We observed that genistein treatment at 0.25% in the VHF model and 0.2% in the HF plus MGO model significantly inhibited BW gain by 88% and 75%. However, the BW gain was inhibited by 41.2% by 0.2% genistein treatment in obese mice. Moreover, genistein treatment at 0.25% in the VHF model and 0.2% in the HF plus MGO model significantly decreased the levels of plasma glucose, cholesterol, ALT, and AST and

liver TG induced by VHFD and HFD plus MGO treatment, respectively. However, none of these markers were significantly decreased by 0.2% genistein treatment in this study. Both the current and our previous studies found that 0.2% and 0.25% genistein significantly decreased liver weight comparing to HFD treated mice, which is also consistent with previous reports.[7, 40, 42, 44-45] Interestingly, the weight of Ep fat was increased in G 0.2 group mice compared with the HF group. The similar effect of the increase of Ep fat was also found at the doses ranging from 50 μg/kg/d to 50, 000 μg/kg/d (50 mg/kg/d) and in dietary genistein at 800 ppm (0.08% in diet) in the short-term genistein administration studies in mice,[48] which are different to most other genistein obesity studies.

Altogether, dietary genistein exerted certain beneficial effects in the HFD pre-induced obese mice, albeit less potent to HFD treated young healthy mice. This is potentially due to it is always easier to prevent the development of a disease than to treat an existing diease. To healthy mice, genistein can efficiently inhibit HFD-induced MGO and AGEs accumulation and body weight gain at the early stage. While to obese mice, MGO and AGEs have already been accumulated, whichh will be much more difficult to be reversed by genistein treatment. In addition, our previous studies started with 6-week old mice for a 16-week treatment and this study stared genistein treatment with 15-week old mice for a 19-week treatment. Therefore, age could be a tributing factor to the observed differences between this study and our previous studies.

In conclusion, the present study demonstrated that dietary genistein intervention in obese mice significantly decreased HFD-induced body weight gain, inhibited the accumulation of MGO and AGEs *via* trapping MGO and activating MGO detoxification pathways, and blocked the AGE/RAGE pathway, albeit the beneficial effects of genistein in obese mice are not as significant as those in the young healthy mice. Furthermore, the findings from our present and previous studies

suggest that genistein may be a candidate agent for alternative or complementary treatment in unhealthy food-induced MetS. Therefore, it is worthwhile to further study the beneficial effects of genistein in human trials.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AGEs, advanced glycation end products; ALT, alanine aminotransferase; AR, aldose reductase; AST, aspartate aminotransferase; Ep fat: epididymal fat; FFA: free fatty acid; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GLO, glyoxalase; HDL, high-density lipoproteins; HFD, high-fat diet; LDL, low-density lipoprotein; MGO, methylglyoxal; MetS, metabolic syndrome; RAGE, the receptor for AGEs; Rp fat: retroperitoneal fat; SIM, selective ion monitoring; VHFD, very-high-fat diet.

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462 **Table 1.** Nutrient composition of HF diet, 0.1% and 0.2% genistein in HF diet[1]

	HF(D12451)	0.1% G (G 0.1)	0.2% G (G 0.2)
Macronutrients [2]			
Protein	20	20	20
Carbohydrate	35	35	35
Fat	45	45	45
Total	100	100	100
Ingredient [3]			
Casein/80 mesh	200	200	200
L-cystine	3	3	3
Corn starch	72.8	72.8	72.8
Maltodextrin 10	100	100	100
Sucrose	172.8	172.8	172.8
Cellulose, BW200	50	50	50
Soybean oil	25	25	25
Lard	177.5	177.5	177.5
Mineral mix[4], S10026	10	10	10
DiCalcium Phosphate	13	13	13
Calcium carbonate	5.5	5.5	5.5
Potassium citrate, $1H_2O$	16.5	16.5	16.5
Vitamin mix[4], V10001	10	10	10
Choline Bitartrate	2	2	2
Genistein	0	0.86	1.72
Dye (different color)	0.05	0.05	0.05
Total	858.15	859.01	859.87
Genistein (%)	**0**	**0.1**	**0.2**

[1] Diets were prepared by Research Diets (New Brunswick, NJ).

[2] Values are presented as the percent of energy in the diet (kcal%).

[3] Values are expressed as g/kg of diet.

463 [4] Complete vitamin and mineral mixture compositions are described previously [49].

FIGURE LEGENDS

Figure 1. Outline of study design. Six-week-old male C57BL/6J mice were fed with a high-fat diet (HFD, 45% energy from fat) for 9 weeks to induce obesity. Then the obese mice were divided into three groups (WK 0) and given the special diets of HF, G 0.1% in HFD, and G 0.2% in HFD for 19 weeks. At the end of the study (WK 19), the mice were sacrificed, plasma and tissue samples were collected for later analysis. G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; HFD, high-fat diet; WK, week.

Figure 2. Effects of genistein supplementation on body weight, food intake, and tissue weights of mice fed the HFD, HFD supplemented with 0.1% (G 0.1), and HFD supplemented with 0.2% genistein (G 0.2) for 19 weeks. The body weight was monitored every week (A) and the cumulative daily food consumption was calculated at the termination of experiment (B). Major organs were weighed after mice were sacrificed (C). Data are shown as means ± SD, n=10. Labeled by different letters are significantly different, $p < 0.05$. Ep fat: epididymal fat; G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; HFD, high-fat diet; Rp fat: retroperitoneal fat.

Figure 3. Effects of genistein supplementation on fasting blood glucose, plasma AST and ALT, plasma TG and cholesterol, and liver TG of mice fed the HFD, HFD supplemented with 0.1% (G 0.1), HFD supplemented with and 0.2% genistein (G 0.2) for 19 weeks. Data are shown as means ± SD, n=10. Labeled by different letters are significantly different, $p < 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; HFD, high-fat diet; TG, triglyceride.

Figure 4. Effects of genistein supplementation on the MGO and AGEs concentrations in plasma, liver, and kidney of mice fed the HFD, HFD supplemented with 0.1% (G 0.1), HFD supplemented with and 0.2% genistein (G 0.2) for 19 weeks. Data are shown as means ± SD, n=10. Labeled by

different letters are significantly different, $p < 0.05$. AGEs, advanced glycation end products; G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; HFD, high-fat diet; MGO, methylglyoxal.

Figure 5. Protein expressions of GLO I and II, GCLC and GCLM, AR, and RAGE in the kidney of mice fed the HFD, HFD supplemented with 0.1% (G 0.1), HFD supplemented with and 0.2% genistein (G 0.2) for 19 weeks (A). The immunoblot bands were quantified by densitometry analysis by ImageJ, and the ratio of β-actin was calculated by setting the value of HF as 1 (B). Data are shown as means ± SD. Labeled by different letters are significantly different, $p < 0.05$. AR, aldose reductase; G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GLO, glyoxalase; HFD, high-fat diet; RAGE, the receptor for AGEs.

Figure 6. Protein expressions of GLO I and II, GCLC and GCLM, AR, and RAGE in the liver of mice fed the HFD, HFD supplemented with 0.1% (G 0.1), HFD supplemented with and 0.2% genistein (G 0.2) for 19 weeks (A). The immunoblot bands were quantified by densitometry analysis by ImageJ, and the ratio of β-actin was calculated by setting the value of HF as 1 (B). Data are shown as means ± SD. Labeled by different letters are significantly different, $p < 0.05$. AR, aldose reductase; G 0.1, 0.1% genistein in HFD; G 0.2, 0.2% genistein in HFD; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GLO, glyoxalase; HFD, high-fat diet; RAGE, the receptor for AGEs.

Figure 7. The LC chromatograms under selected ion monitoring mode and the MS^2 spectra of MGO-GEN (A), MGO-6′-OH-DMA (B) and MGO-DHGEN (C) in mouse feces after feeding with HF diet, HFD supplemented with 0.1% (G 0.1), HFD supplemented with and 0.2% genistein (G 0.2) for 19 weeks, as well as respective references obtained by a negative ESI-MS interface. MGO-

510 GEN, mono-methylglyoxal adducts of genistein. MGO-6′-OH-DMA, mono-methylglyoxal
511 adducts of 6'-hydroxy-*O*-demethylangolensin. MGO-DHGEN, mono-methylglyoxal adducts of
512 dihydrogenistein. HF diet, high-fat diet. HF+G 0.1, HF diet supplemented with 0.1% genistein.
513 HF+G 0.2, HF diet supplemented with 0.2% genistein. SIM, selected ion monitoring. ESI,
514 electrospray ionization.

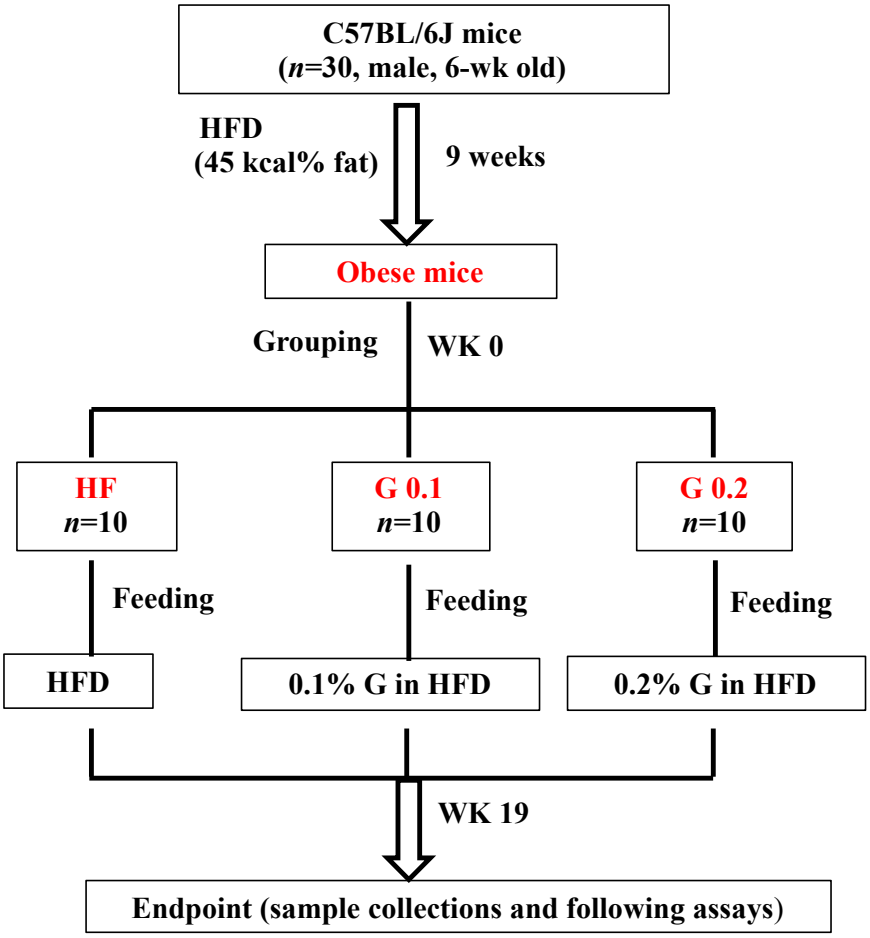

C57BL/6J mice
(n=30, male, 6-wk old)
HFD
(45 kcal% fat)
9 weeks
Obese mice
Grouping
WK 0
HF
n=10
G 0.1
n=10
G 0.2
n=10
Feeding
Feeding
Feeding
HFD
0.1% G in HFD
0.2% G in HFD
WK 19
Endpoint (sample collections and following assays)

Figure 1

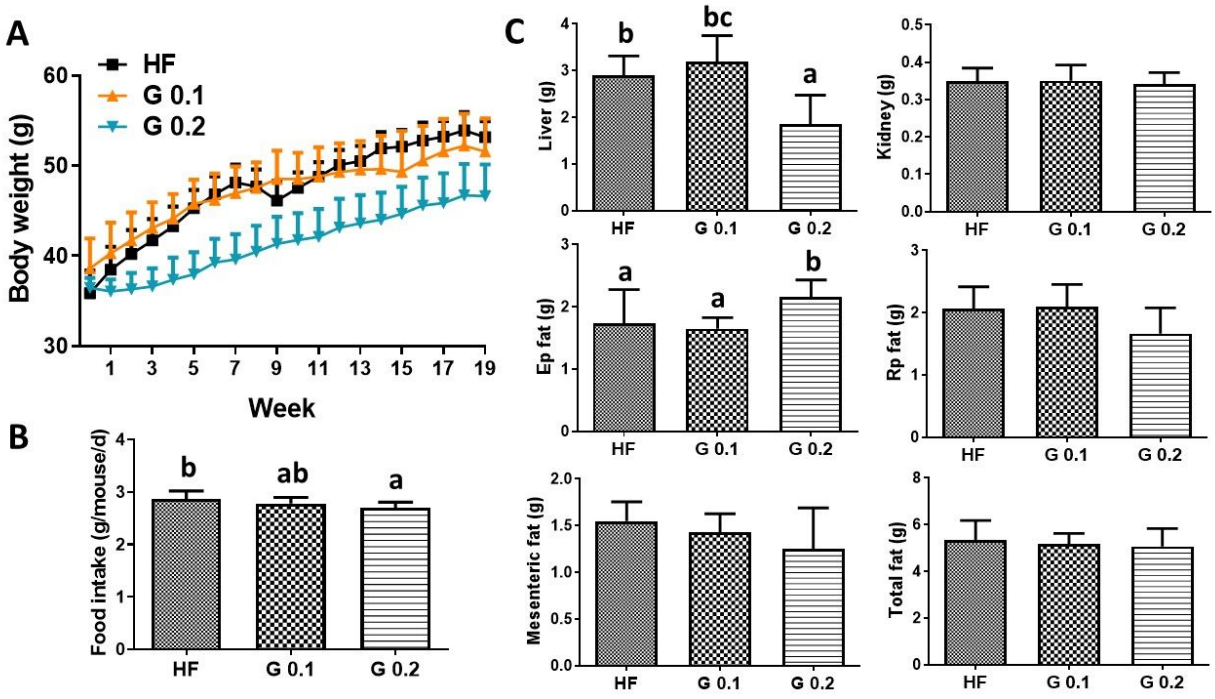

A
HF
G 0.1
G 0.2
Body weight (g)
Week
B
Food intake (g/mouse/d)
C
Liver (g)
Kidney (g)
Ep fat (g)
Rp fat (g)
Mesenteric fat (g)
Total fat (g)

Figure 2

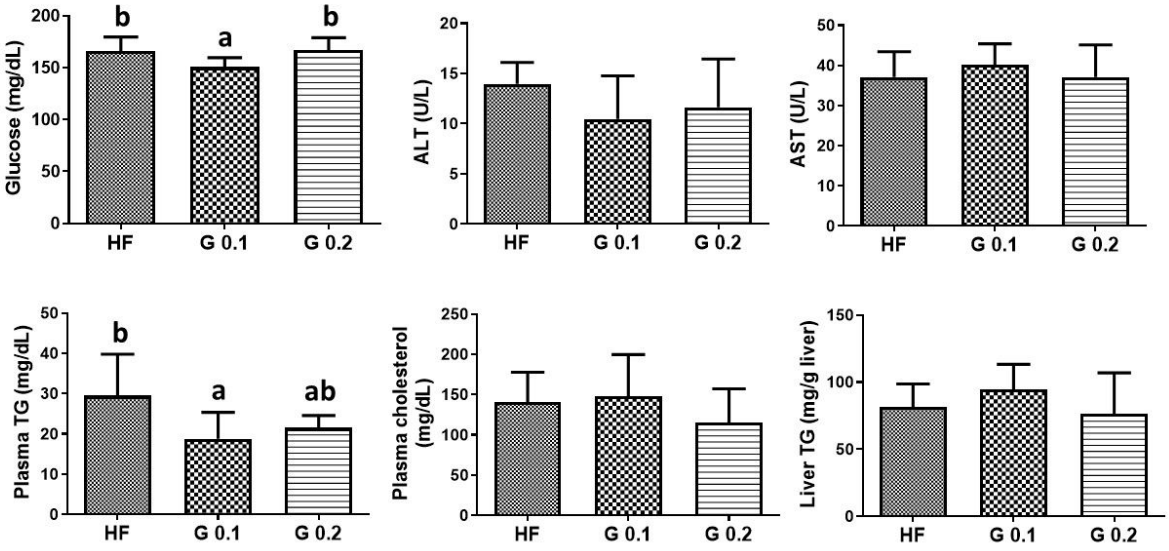

Glucose (mg/dL)
200
150
100
50
0
b
a
b
HF
G 0.1
G 0.2
ALT (U/L)
20
15
10
5
0
HF
G 0.1
G 0.2
AST (U/L)
50
40
30
20
10
0
HF
G 0.1
G 0.2
Plasma TG (mg/dL)
50
40
30
20
10
0
b
a
ab
HF
G 0.1
G 0.2
Plasma cholesterol (mg/dL)
250
200
150
100
50
0
HF
G 0.1
G 0.2
Liver TG (mg/g liver)
150
100
50
0
HF
G 0.1
G 0.2

Figure 3

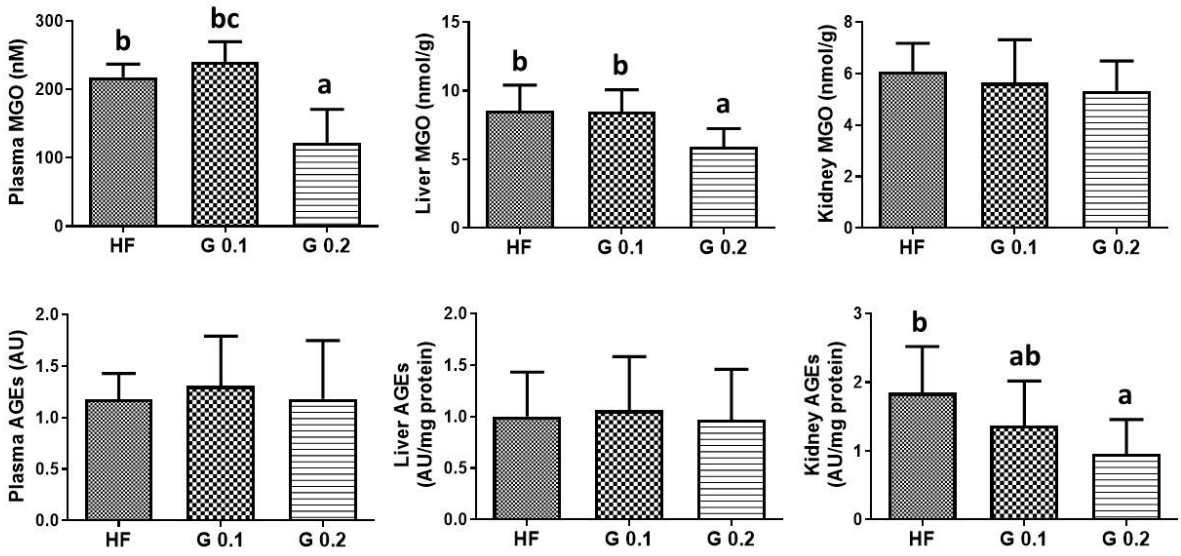

Plasma MGO (nM)
300
200
100
0
b
bc
a
HF
G 0.1
G 0.2
Liver MGO (nmol/g)
15
10
5
0
b
b
a
HF
G 0.1
G 0.2
Kidney MGO (nmol/g)
8
6
4
2
0
HF
G 0.1
G 0.2
Plasma AGEs (AU)
2.0
1.5
1.0
0.5
0.0
HF
G 0.1
G 0.2
Liver AGEs (AU/mg protein)
2.0
1.5
1.0
0.5
0.0
HF
G 0.1
G 0.2
Kidney AGEs (AU/mg protein)
3
2
1
0
b
ab
a
HF
G 0.1
G 0.2

Figure 4

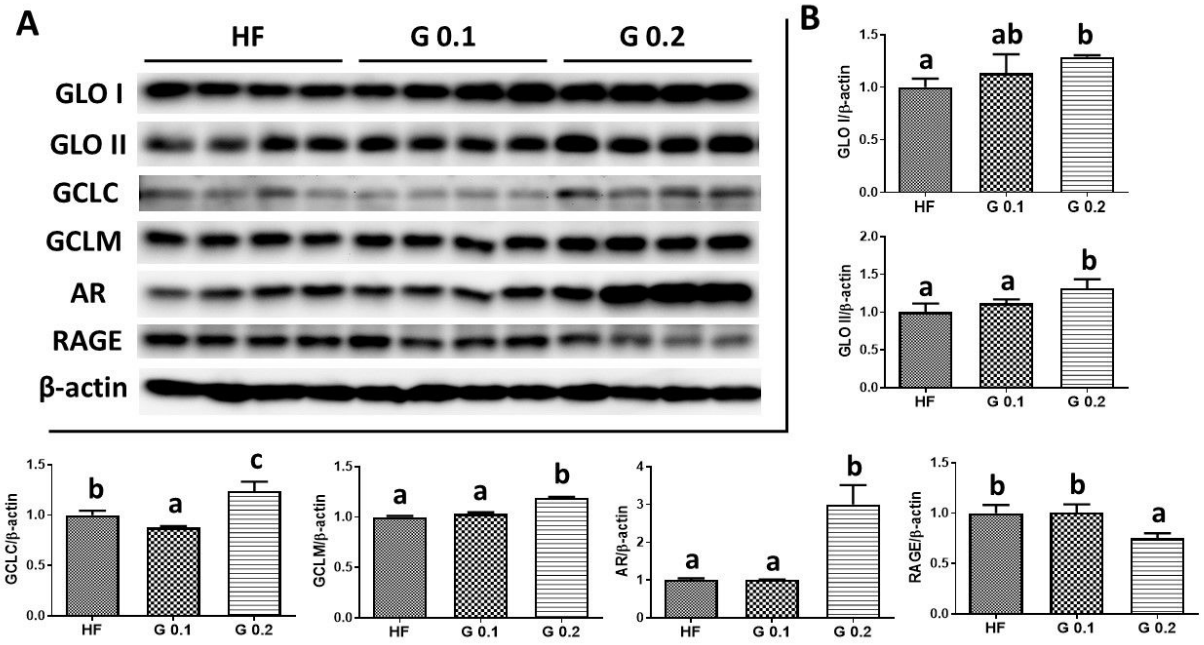

A
HF
G 0.1
G 0.2
GLO I
GLO II
GCLC
GCLM
AR
RAGE
β-actin
B
GLO II/β-actin
GLO II/β-actin
GCLC/β-actin
GCLM/β-actin
AR/β-actin
RAGE/β-actin

Figure 5

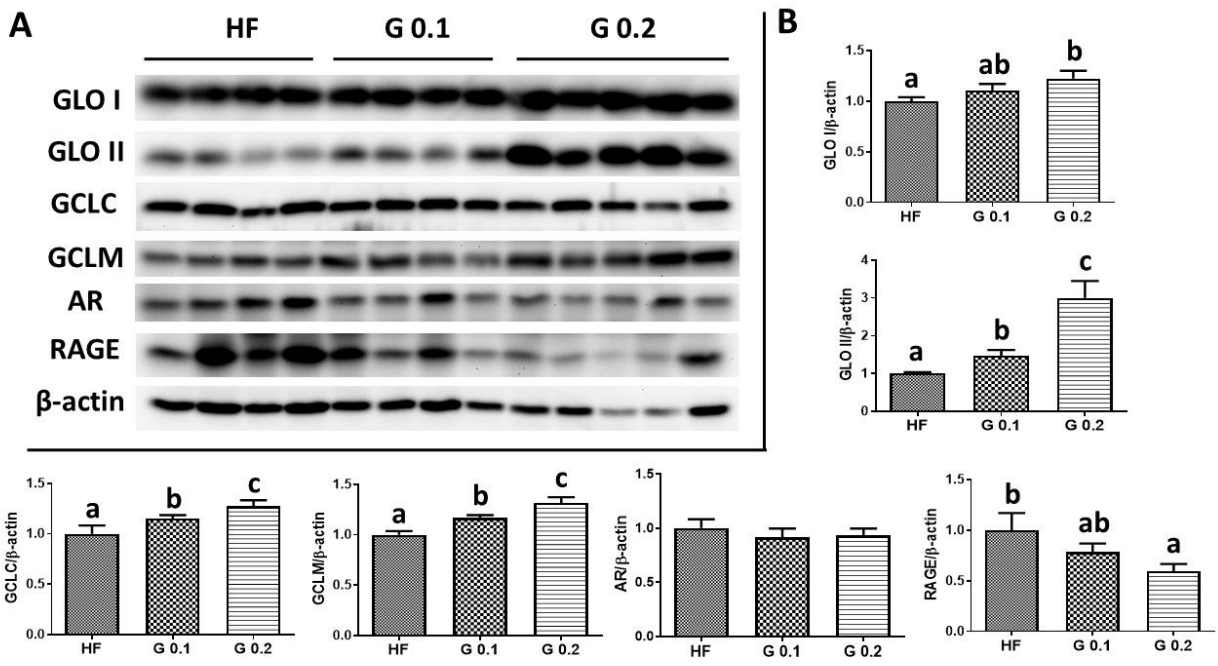

A
HF
G 0.1
G 0.2
GLO I
GLO II
GCLC
GCLM
AR
RAGE
β-actin
B
GLO I/β-actin
GLO II/β-actin
GCLC/β-actin
GCLM/β-actin
AR/β-actin
RAGE/β-actin
a
ab
b
c

Figure 6

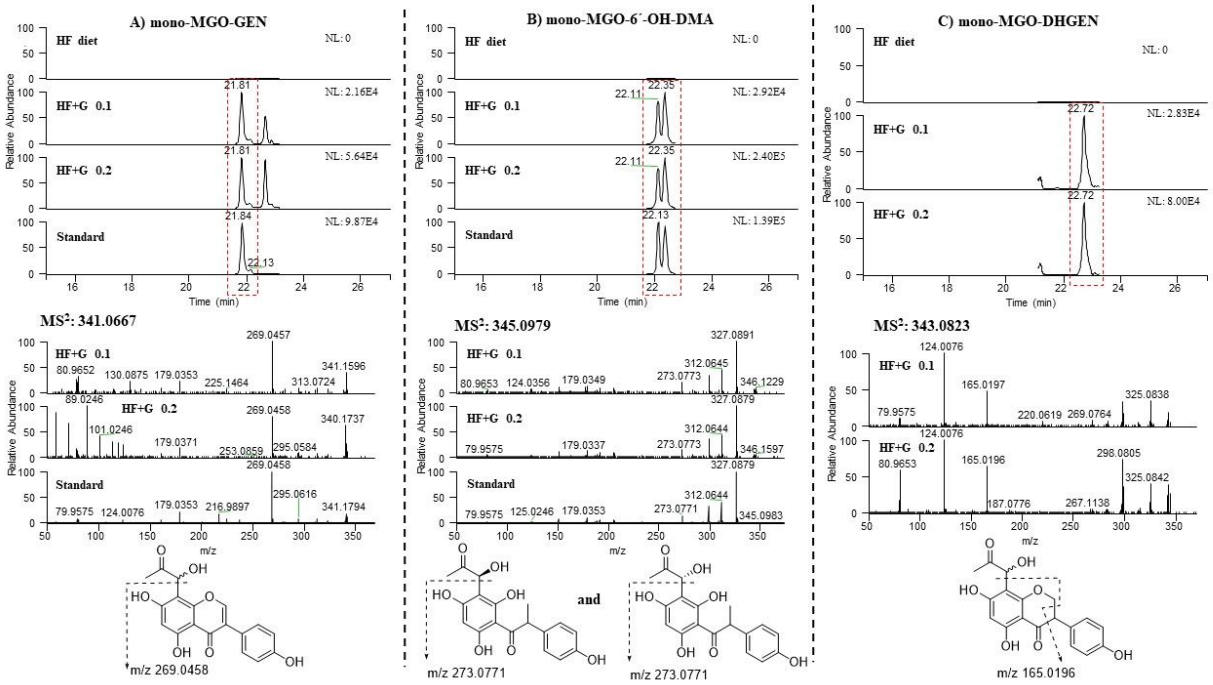
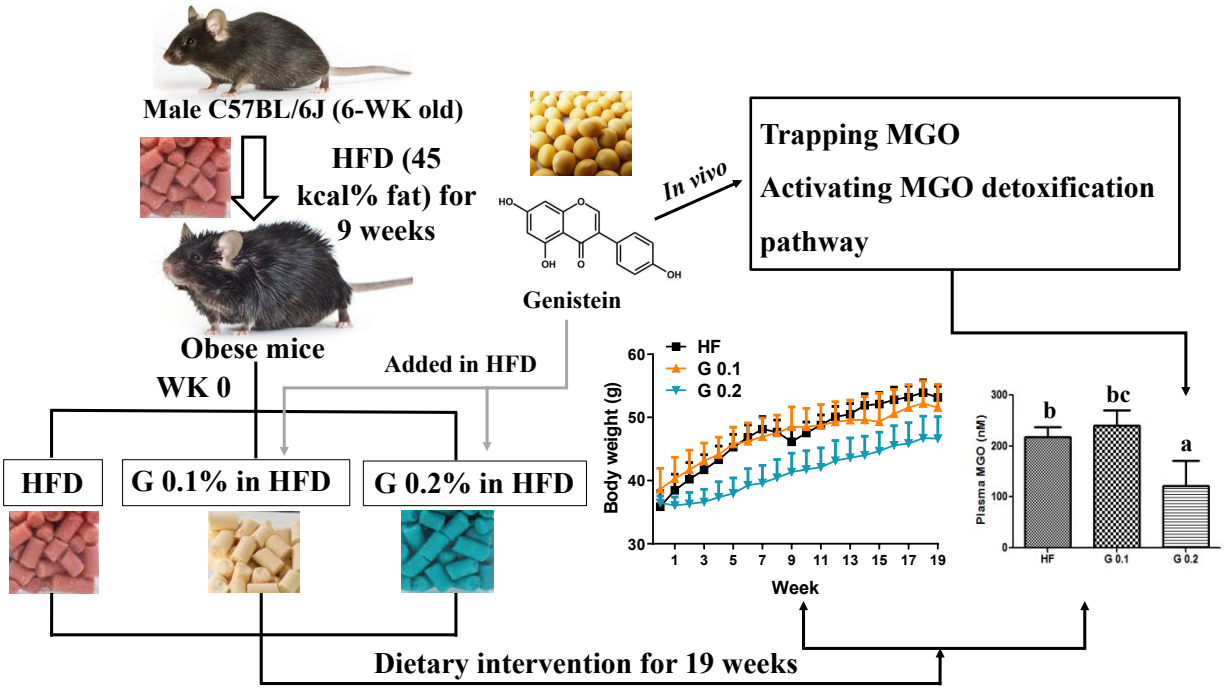

A) mono-MGO-GEN
B) mono-MGO-6´-OH-DMA
C) mono-MGO-DHGEN
HF diet
HF+G 0.1
HF+G 0.2
Standard
NL: 0
NL: 2.16E4
NL: 5.64E4
NL: 9.87E4
NL: 2.92E4
NL: 2.40E5
NL: 1.39E5
NL: 2.83E4
NL: 8.00E4
Relative Abundance
Time (min)
MS²: 341.0667
MS²: 345.0979
MS²: 343.0823
m/z
and
m/z 269.0458
m/z 273.0771
m/z 273.0771
m/z 165.0196

Figure 7

TOC Graphic


Male C57BL/6J (6-WK old)
HFD (45 kcal% fat) for 9 weeks
Obese mice
WK 0
Genistein
In vivo
Trapping MGO
Activating MGO detoxification pathway
Added in HFD
HFD
G 0.1% in HFD
G 0.2% in HFD
HF
G 0.1
G 0.2
Body weight (g)
Week
Plasma MGO (nM)
b
bc
a
Dietary intervention for 19 weeks