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Original Article

Genistein in combination with Chlorogenic acid ameliorates insulin resistance and oxidative stress in skeletal muscle of high-fat diet fed mice

Golnaz Goodarzi[1], Mitra Yadollahi [2], Sadra Samavarchi Tehrani[3], Reza Meshkani[4]

[1] *Department of Pathobiology and Laboratory Sciences, North Khorasan University of Medical Sciences, Bojnurd, Iran.*
[2] *Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran*
[3] *Endocrine Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Science, Tehran, Iran*
[4] *Department of Clinical Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, I.R Iran.*

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* **Corresponding Author:**

Reza Meshkani,
Department of Clinical Biochemistry,
Faculty of Medicine,
Tehran University of Medical Sciences, Tehran, I.R. Iran.

Email: rmeshkani@tums.ac.ir

ABSTRACT

Objectives: Increasing evidence has demonstrated that oxidative stress plays a significant role in the pathogenesis of muscle insulin resistance. The beneficial impacts of genistein (GEN) and chlorogenic acid (CGA) on insulin resistance have already been revealed. However, their combined effects on skeletal muscle oxidative stress and insulin resistance have not been completely understood. The aim of the present study was to determine the effect of GEN in combination with CGA on skeletal muscle insulin resistance in high-fat diet (HFD) fed C57BL/6 mice.

Methods: C57BL/6 male mice were fed an HFD for 15 weeks. The mice were then divided into five groups: standard chow diet (SCD), HFD, HFD + GEN, HFD + CGA, and HFD + GEN + CGA for 10 weeks.

Results: The findings indicated that single treatment with GEN or CGA, and with a stronger effect of GEN+CGA combined treatment, decreased body weight gain and improved glucose intolerance. Moreover, following treatment with GEN and CGA alone or in combination with further effect, the level of plasma and muscle triglyceride (TG) were reduced. Furthermore, the combination therapy of GEN and CGA with greater efficacy than the single treatments, could decrease several oxidative stress markers in the skeletal muscle. Treatment with GEN and CGA alone or in combination could increase the expression of NF-E2–related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NAD(P): Quinone Oxidoreductase 1 (NQO1).

Conclusion: The data of this study suggest that the combination of GEN and CGA might ameliorate insulin resistance and reduce oxidative stress in the skeletal muscle of mice fed HFD.

Keywords: Genistein, Chlorogenic Acid, Oxidative Stress, Insulin Resistance, Skeletal Muscle, Nrf2

Abbreviations: CGA: and chlorogenic acid; FBS: Fasting blood glucose; FRAP: ferric reducing antioxidant power; HFD: high fat diet; GEN: genistein; HO-1: heme oxygenase-1; IPGTT: Intraperitoneal glucose tolerance test; MDA: malondialdehyde ; NAFLD: non-alcoholic fatty liver disease; SCD: standard chow diet; NQO1: NAD(P): Quinone Oxidoreductase 1; Nrf2: NF-E2–related factor 2; ROS: reactive oxygen species; TG: triglyceride; T2DM: Type 2 diabetes mellitus.

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Introduction

Type 2 diabetes mellitus (T2DM) and obesity are now considered common chronic disorders. It is well accepted that obese individuals are at risk of developing diabetes (1). Despite extensive research on the molecular mechanisms underlying T2DM, its exact pathophysiology is not fully understood (2). Skeletal muscle is the main location for postprandial glucose elimination, and thus it is regarded as one of the main tissues involved in the pathogenesis of T2DM (3, 4).

Oxidative stress plays an important role in the pathogenesis and progression of T2DM (5). Oxidative stress is defined as an imbalance between the production of free radicals and the activity of the antioxidant defense system. Many factors have been reported to induce oxidative stress in various tissues (6). Among them, high free fatty acids and glucose levels could stimulate the excessive production of mitochondrial reactive oxygen species (ROS), leading to increased oxidative stress in the skeletal muscle (7). Oxidative stress can then impair the insulin signaling pathway by activating several protein kinases (PK) such as PKC, followed by serine phosphorylation of the insulin receptor in the skeletal muscle (8). In addition, the excessive ROS production can change cellular components such as proteins, lipids, and DNA, resulting in injury to different tissues (9). To counter the detrimental effect of oxidative stress, the body activates the antioxidant defense system to remove excess ROS and avoid oxidative damage. Increasing the activity of the antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P): Quinone Oxidoreductase 1 (NQO1), and superoxide dismutase (SOD) has been reported to be able to reduce the severity of oxidative damage through controlling the amount of ROS (10). In this regard, oxidative stress reduction has been considered as a therapeutic solution by many researchers in recent years.

Emerging evidence has shown that natural compounds with antioxidant properties improve the complications associated with obesity and T2DM by reducing the damages caused by oxidative stress (11-15). Genistein (GEN), an isoflavone phytoestrogen from the flavonoid family of polyphenol, is found in soybeans and leguminous plants (16). GEN has received considerable attention due to its numerous pharmacological features including anti-cancer, anti-oxidant, anti-osteoporosis, and anti-inflammatory effects (17-19). Many experimental studies have revealed that this phenolic agent could decrease oxidative stress markers and also hepatic lipid accumulation in high-fat diet (HFD)-induced non-alcoholic fatty liver disease (NAFLD) model (20-22). In another study, it was indicated that GEN decreased the oxidative stress caused by hyperglycemia in the kidney tissue of diabetic rats by significantly reducing the amount of malondialdehyde (MDA) and enhancing nuclear factor erythroid 2–related factor 2 (Nrf2) activity, a vital regulator of the cellular resistance to oxidants (23). Another important polyphenolic compound belonging to the hydroxycinnamic acid family is chlorogenic acid (CGA) (24). In recent years, researchers have focused on the wide range of health benefits of this natural compound in the treatment of metabolic syndrome, diabetes, obesity, and NAFLD (25, 26). In the study conducted by Ye et al., it was demonstrated that CGA reduced oxidative stress in streptozotocin-induced diabetic nephropathy rats by increasing the activity of antioxidant enzymes through regulating the Nrf2/keap1 pathway (27). Both compounds have been reported to attenuate insulin resistance in different tissues, however, the beneficial effects of the combination of GEN and CGA on insulin resistance and more specifically oxidative stress in the skeletal muscle is unknown. Therefore, this study aimed to investigate the effects of combined therapy of CGA and GEN on insulin resistance and oxidative stress induced by HFD in the skeletal muscle of C57BL/6 mice.

Material and Methods

Animals, diet and sample preparation

For this study, seven-week-old male C57BL/6 mice were obtained from the Pasteur Institute of Iran and housed in cages in standard conditions with a 12:12 h light-dark cycle, relative humidity (50%), and temperature (22±2 °C). After a one-week adaptation period, the mice were randomly divided into two groups based on the diets for 15 weeks as follows: 10 mice were placed in the standard chow diet (SCD) group containing 10 kcal% fat, and the other mice (n=40) received an HFD that contained 60 kcal% fat. After this time, the group fed an HFD was separated into four groups, each group consisting of 10 mice, and the treatment continued for another 9 weeks as defined: HFD, HFD plus 0.02% CGA (0.2 g/kg diet), HFD plus 0.2% GEN (2 g/kg diet), HFD plus 0.02% CGA and 0.2% GEN. It is of note that the doses of CGA and GEN used in this study were obtained from previous studies (28, 29). The body weight of the mice was measured at the beginning of the study; it was checked weekly along with food intake during the treatment period.

At the end of the study and after six hours of fasting, the animals were sacrificed by intraperitoneal injection of a ketamine-xylazine mixture. To measure biochemical parameters, plasma was separated from blood samples taken from their heart and finally stored at -80 °C. The quadriceps skeletal tissue was quickly excised and washed to remove excess blood; a part of the tissue was frozen in liquid nitrogen and kept at -80 °C for assessing molecular analysis and checking oxidative stress factors.

All the experimental procedures of the present study were performed based on the guidelines approved by the Ethics Committee of Tehran University of Medical Sciences.

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was conducted to evaluate glucose tolerance in animals. In the final week of the treatment period, this test was performed following a 6-hour overnight fast. Blood glucose was assessed in samples taken from the tail vein before the intraperitoneal injection of glucose (2 g/kg) and at 15, 30, 60, 90, and 120 min after injection by the Accu-check Aviva system (Roche Diagnostics, Burgess Hill, UK). The Area Under the Curve (AUC) was applied to evaluate the severity of reduced glucose tolerance.

Analysis of biochemical markers

The concentration of triglyceride (TG) and fasting blood glucose (FBG) in plasma were determined using biochemical kits (Pars Azmon kit, Iran) and an auto-analyzer system.

Measurement of skeletal muscle TG

The lipid content of skeletal muscle tissue was measured based on the Folch method (30). Tissues were homogenized through ultrasonication after rinsing in phosphate buffer saline (PBS). The homogenates were centrifuged at 12000 g for 20 min at 4 °C. The extracted lipids were detected by a colorimetric assay kit (Pars Azmon, Iran).

Measurement of oxidative stress parameters

The Ferric Reducing Antioxidant Power (FRAP) assay was utilized to investigate the effect of combined treatment of CGA and GEN on the antioxidant power of reducing ferric-tripyridyltriazine (TPTZ) to ferrous-TPTZ form in low pH by muscle homogenates samples. The absorbance of the produced blue color was read at a wavelength of 593 nm (31, 32). A standard curve was prepared to determine the concentration of Fe+2 by adding ferrous sulfate with a concentration range from 0-2 mM to the FRAP solution. Total protein content in tissues was measured using a BCA kit. Results were shown as µmol/mg tissue protein.

The colorimetric method described by Sedlak and Lindsay (33) was utilized, using Ellman's reagent (5,5-dithionitrobenzoic acid) to evaluate the total thiol content in the homogenized muscle tissue. The OD yellow-colored product was quantified at 412 nm. Thiol concentration is calculated based on µmol/mg tissue protein.

The concentration of malondialdehyde (MDA) in muscle homogenates, a known marker of oxidative damage to lipids, was assessed. Homogenized tissue was exposed to the solution containing 0.375% thiobarbituric acid (TBA), 0.25N hydrochloric acid (HCL), and 15% trichloroacetic acid (TCA) for 30 minutes, and then the mixtures were heated at 100 °C. After cooling, the mixtures were centrifuged and the optical absorbance of the pink-colored product was calculated at 535 nm (34). The concentration of MDA was obtained using the standard curve prepared with tetramethoxypropane and the results were shown as nmol/mg tissue protein.

The level of protein carbonyl (an indicator of protein peroxidation) was evaluated in muscle homogenates using a method proposed by Picker and Reznick (35). Based on this method, protein hydrazones are formed in the reaction of 2,4-dinitrophenylhydrazine and protein carbonyl groups. The absorbance of the product was measured at 370 nm. Results were reported as nmol/mg tissue protein.

RNA extraction and qRT-PCR analysis

The total RNA from the skeletal muscle tissue sample was isolated using the Ana-Cell Super RNA extraction kit (Ana Cell tec, Iran), following the manufacturer's protocol. The quality and purity of the extracted RNAs were determined by a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The Ana Cell cDNA synthesis kit (Ana Cell, Iran) was utilized to convert RNA to cDNA. A quantitative real-time polymerase chain reaction (qRT-PCR) assay was carried out to check the expression level of Nrf2, HO-1, and NQO1 in each group via SYBR Green RealQ Plus 2X Master Mix Green (amplicon), cDNA, and specific primers of genes (Table 1, in the supplementary file) on Rotor-gene (Qiagene, Hilden, Germany). β-actin was used as a reference gene for the normalization of the data based on the Delta-Delta method.

Statistical analysis

SPSS version 20.0 (IBM Corporation., Armonk, NY, USA) and GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis. All data are presented as mean ± SEM. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A value of $P<0.05$ was regarded statistically significant.

Results

Combination of CGA and GEN reduced body weight in HFD fed mice

To assess the impact of CGA alone and in combination with GEN on body weight in mice fed with HFD,

measurements of body weight were taken throughout the study and at the conclusion of 25 weeks. As anticipated, the group of mice on the HFD displayed a significant increase in body weight compared to the SCD group from week 5 until the end of the study. Both CGA and GEN were able to reduce body weight compared to the HFD, although not significantly. However, the combined therapy of CGA and GEN significantly reduced body weight compared to the individual treatments (Fig. 1a, and b). Furthermore, no significant differences were noted in the average daily food intake among the groups during the experiment (Fig 1c).

Combination of CGA and GEN improved insulin resistance in HFD fed mice

To investigate the effects of CGA and GEN, both individually and in combination, on glycemic status, FBS and IPGTT tests were conducted. As depicted in (Fig. 2a), supplementation therapy with CGA and CGA+GEN significantly reduced FBS compared to the HFD group. Monotherapy with GEN did not affect FBS. However, the combination of GEN and CGA resulted in a greater reduction in FBS compared to monotherapy with GEN and CGA.

To verify the effects of combination therapy with CGA and GEN on insulin resistance, an IPGTT was performed. As shown in (Fig 2c), no significant difference was observed between the single treatments of CGA and GEN compared to HFD on AUC. However, the combination therapy with CGA and GEN resulted in a lower AUC than the monotherapies with GEN and CGA, as well as the HFD groups.

Combination of CGA and GEN reduced TG content in skeletal muscle of HFD treated mice

As shown in (Fig. 3a, and b), the level of plasma TG and muscle TG content showed an increase in the HFD group when compared to the SCD. Both CGA and GEN, individually and in combination, significantly reduced the concentration of TG in plasma and muscle. Notably, the combination therapy demonstrated a greater potential to decrease the concentration of TG in plasma and skeletal muscle compared to monotherapy.

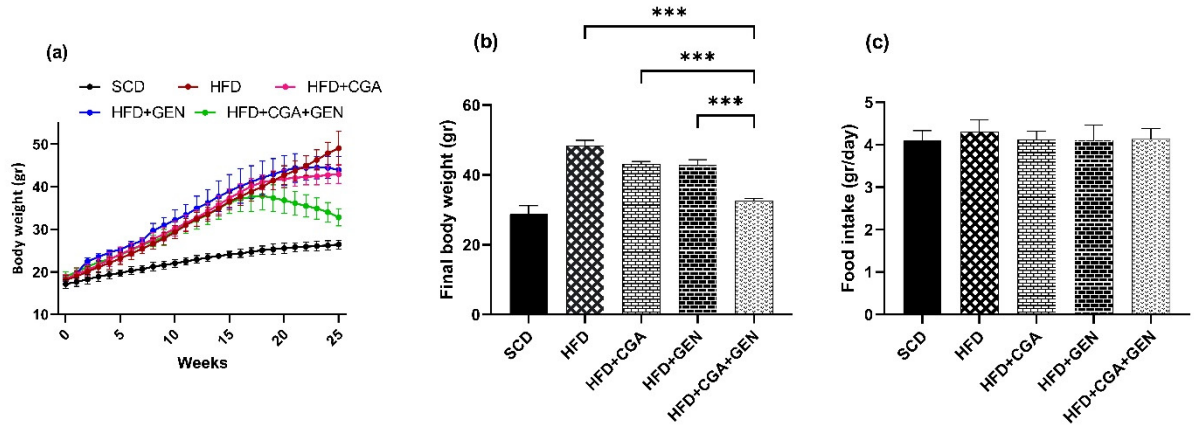

Figure 1.: The effects of CGA+GEN supplementation in HFD- fed mice. Body weight gain in period of 25 weeks(a), final body weight at the end of experiment (b), and food intake(c). Data are presented as means±SEM; n=10. Values are significantly different between groups as determined using one-way ANOVA (*p < 0.05, **p < 0.01, and ***p < 0.001)

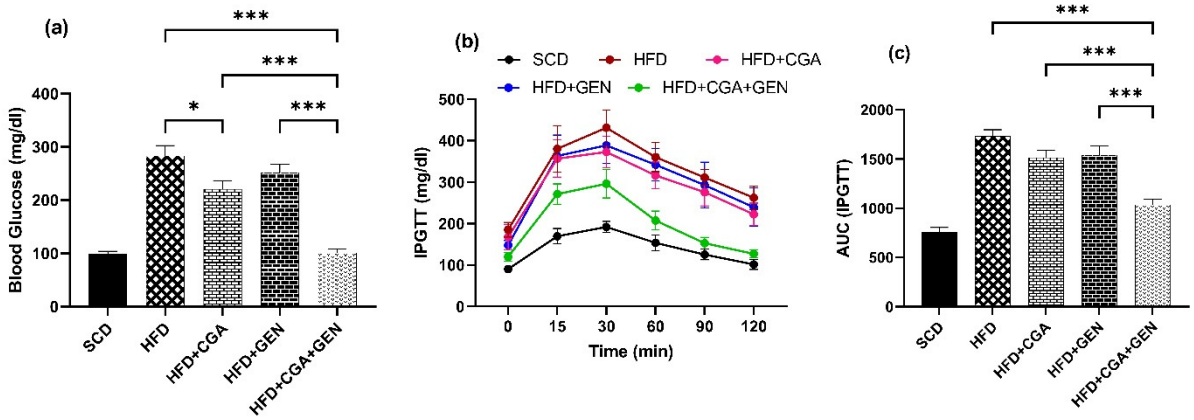

Figure 2: Effects of CGA+GEN supplementation on fasting blood glucose level (a), and iPGTT(b), AUC (c). Data are presented as mean ±SEM. Values are significantly different between groups as determined using one-way ANOVA. (*p < 0.05, **p < 0.01, and ***p < 0.001).

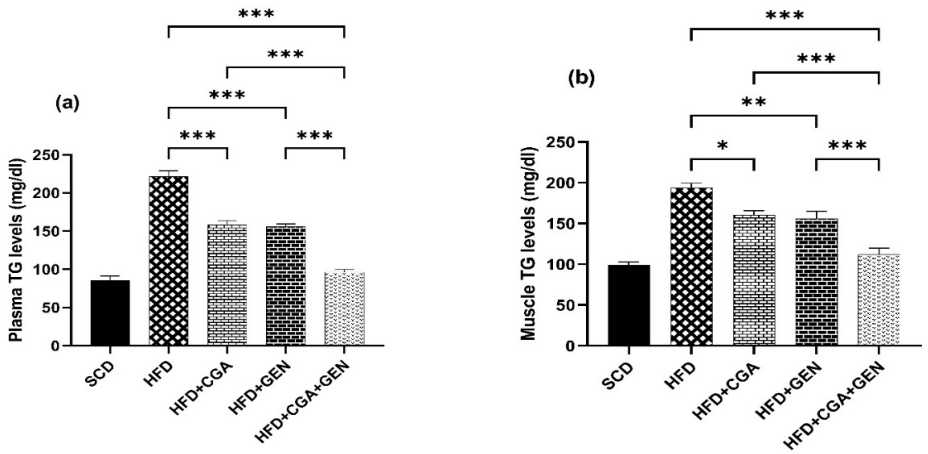

Figure 3: The effects of CGA+GEN supplementation on TG content in HFD fed mice. TG plasma level (a), and TG content in muscle (b). Data are presented as means±SEM; n=10. Values are significantly different between groups as determined using one-way ANOVA (*p < 0.05, **p < 0.01, and ***p < 0.001).

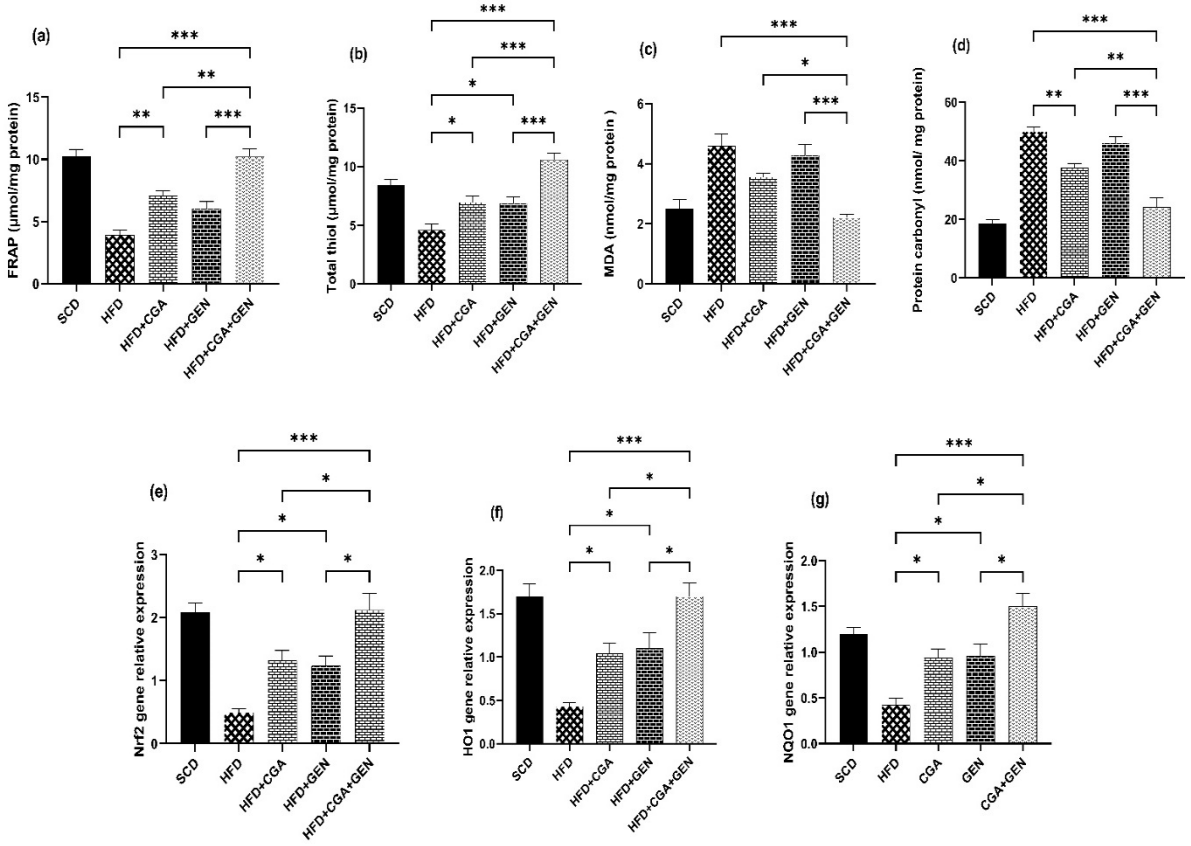

Figure 4: Combined treatment of CGA with GEN reduces oxidative stress induced by HFD. Ferric reducing antioxidant power (FRAP) (a), total thiol content (b), malondialdehyde (MDA) levels (c), protein carbonyl content(d), mRNA expression of Nrf2 (e), mRNA expression of HO-1 (f), and mRNA expression of NQO1 (g). Data are presented as means±SEM; n=10. Values are significantly different between groups as determined using one-way ANOVA (*p < 0.05, **p < 0.01, and ***p < 0.001).

Combination of CGA and GEN alleviated oxidative stress-related parameters in HFD fed mice

To investigate the antioxidant capacity of CGA and GEN in the muscle of HFD-fed mice, FRAP, total thiol content, MDA, and protein carbonyl tests were performed. As shown in Fig. 4, FRAP and total thiol content were strongly decreased in the HFD group. Monotherapy with CGA showed a significant increase in FRAP and total thiol content compared to the HFD

group. However, GEN monotherapy had no effects on these parameters, but GEN in combination with CGA has more potential to increase FRAP and total thiol content compared to treatment with CGA monotherapy. In addition, the results showed a significant elevation of MDA in the HFD group compared to the SCD group. However, MDA level in CGA and GEN monotherapy showed no significant decrease, but combination therapy of CGA and GEN had more potential to decrease MDA level. These results suggested that combination therapy could reverse lipid peroxidation in the skeletal muscle of the mice fed HFD (Fig 4c). Furthermore, CGA+GEN showed the ability to overcome elevated protein carbonyl induced by HFD (Fig 4d).

Nrf2, a master regulator of the antioxidant defense system, protects the cells against oxidative stress through activating its target genes such as NQO1 (36, 37). To investigate the mechanism by which CGA+GEN could ameliorate oxidative stress in the skeletal muscle, the expression of the Nrf2, HO-1, and NQO1 genes in treatment groups was evaluated. As illustrated in Fig 4e, f, and g, the expression of Nrf2, HO-1, and NQO1 were significantly reduced following HFD treatment compared to SCD, whereas treatment with CGA and GEN could significantly reverse the HFD repressive effect. Interestingly, the combined treatment of CGA and GEN had a greater effect than the single treatments. Overall, these results suggest that treatment with CGA in combination with GEN has more potential to prevent HFD-induced oxidative stress in the skeletal muscle.

Discussion

There is ample evidence on the role of oxidative stress in the pathogenesis of chronic diseases such as T2DM (36, 38), and therefore strategies based on the inhibition of oxidative stress have attracted the attention of many researchers. Considering the drawbacks associated with synthetic drugs, there is an increased interest in the consumption of phenolic compounds due to their lower cost and side effects. These compounds also have a free radical scavenging property that protects the cells against oxidative damages (36, 39, 40). In the current study, GEN and CGA, two important natural products with antioxidant effects were chosen due to their beneficial effects on obesity and its related diseases (20, 41-43). To the best of the knowledge, no research has specifically focused on the effect of the combined treatment of CGA with GEN on oxidative stress in the skeletal muscle.

It has been well established that C57BL/6J mice fed HFD is a proper model for studying human metabolic abnormalities associated with obesity (44). In this study, this model was used and the results obtained from plasma level of FBG and AUC of IPGTT indicated that the combination therapy with CGA and GEN improves insulin resistance in HFD mice more effectively than monotherapy of GEN and CGA. It was found when the treatment with CGA and GEN started, in the groups receiving CGA, GEN alone and CGA+GEN without changing in food intake had a reducing effect on body weight, but the effectiveness of c ombined treatment was more. It is noteworthy that the effects of CGA and GEN alone on maintaining glycemic control, helping to reduce body weight and improving insulin resistance have been previously reported (16, 41, 45, 46).

When the amount of free fatty acids is greater than the capacity of the adipocytes to absorb, lipids overflow to non-adipose tissue such as heart, liver and skeletal muscle. Incomplete fat metabolic products, such as ceramides, diacylglycerols (DAGs) are produced when lipid accumulation in the skeletal muscle exceeds the oxidation capacity, and these products inhibit important molecules in the insulin signaling such as Akt, insulin Receptor Substrate (IRS) leading to insulin resistance in skeletal muscle (47). In the current project, the anti-hyperlipidemic properties of CGA and GEN can be inferred due to the reduction of plasma and skeletal muscle TG levels. Although this effect was stronger in CGA+GEN treatment. In a study conducted by Zamani et al., it has been shown that GEN remarkably improves lipid accumulation in NAFLD in HFD fed mice through decreasing the expression of genes related to fatty acid synthesis and increasing genes associated with fatty acid oxidation (29). In addition, several studies implied that CGA decreases hepatic lipid accumulation in animals with lipid abnormalities (48, 49).

Accumulating evidence has demonstrated that increased levels of ROS have a close correlation with diabetes and obesity. The increased supply of free fatty acids and glucose make mitochondria more active and capable of producing more ROS, as a by-product of the electron transport chain (50). Elevated ROS cause mitochondrial fission and damage to the cell and they have been directly related to insulin resistance in skeletal muscle (51). It has been revealed that Nrf2, under normal conditions, plays a major role in maintaining redox balance by regulating a wide range of antioxidant enzymes such as HO-1, NQO1, and SOD (52). Therefore, the use of an Nrf2 activator can potentially be considered as a therapeutic method to counteract the oxidative stress caused by obesity (53). To determine the relationship between oxidative stress and consumption of CGA and GEN, a set of parameters related to oxidative stress and the expression level of genes involved in oxidative stress were examined. The results obtained from these parameters in skeletal muscle tissue in the combined treatment are expressed in the following: (a) a significant increase in FRAP level and total thiol content indicating antioxidant capacity, (b) a significant decrease in protein carbonyl content and MDA level as oxidative stress markers, © a significant increase in the expression of Nrf2, HO-1 and NQO1. According to these findings, it can be concluded that the combination therapy (CGA+GEN) could lead to the best outcomes for modulating these

oxidative stress parameters in skeletal muscle compared to single treatments. In agreement with the effects of CGA, in a study conducted by Preetha Rani et al., it has been demonstrated that CGA, through the antioxidant activity, significantly attenuates hyperglycemia in H9c2 cells (54). The result of Huang's study showed that the stimulation of antioxidant scavenging enzymes and the inhibition of lipid peroxidation were linked to protective effects of GEN on oxidative stress followed by recovery of liver damage and liver fibrosis induced by alcohol administration in rats (55).

In summary, the findings demonstrated that the combination therapy of GEN and CGA, as natural compounds, can improve obesity-induced insulin resistance in the skeletal muscle via reducing the oxidative stress. Additionally, the combination of these two polyphenols showed better effectiveness in comparison with monotherapy in improving the mentioned factors. Overall, these data propose that GEN in combination with CGA might be regarded as a promising therapeutic strategy to inhibit obesity-linked oxidative stress in skeletal muscle cells.

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Conflict of Interest

The authors have nothing to declare.

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