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# Effects of Exercise Training and Chlorogenic Acid Supplementation on Hepatic Lipid Metabolism in Prediabetes Mice

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**Background:** Since prediabetes is a risk factor for metabolic syndromes, it is important to promote a healthy lifestyle to prevent prediabetes. This study aimed to determine the effects of green coffee (GC), chlorogenic acid (CGA) intake, and exercise training (EX) on hepatic lipid metabolism in prediabetes male C57BL/6 mice.
**Methods:** Forty-nine mice were randomly divided into two groups feeding with a normal diet ($n=7$) or a high-fat diet (HFD, $n=42$) for 12 weeks. Then, HFD mice were further divided into six groups ($n=7$/group): control (pre-D), GC, CGA, EX, GC+EX, and CGA+EX. After additional 10 weeks under the same diet, plasma, and liver samples were obtained.
**Results:** HFD-induced prediabetes conditions with increases in body weight, glucose, insulin, insulin resistance, and lipid profiles were alleviated in all treatment groups. *Acsl3*, a candidate gene identified through an *in silico* approach, was lowered in the pre-D group, while treatments partly restored it. HFD induced adverse alterations of *de novo* lipogenesis- and β oxidation-associated molecules in the liver. However, GC and CGA supplementation and EX reversed or ameliorated these changes. In most cases, GC or CGA supplementation combined with EX has no synergistic effect and the GC group had similar results to the CGA group.
**Conclusion:** These findings suggest that regular exercise is an effective non-therapeutic approach for prediabetes, and CGA supplementation could be an alternative to partially mimic the beneficial effects of exercise on prediabetes.

**Keywords:** Chlorogenic acid; Coffee; Exercise; Lipogenesis; Liver; Prediabetic state

## INTRODUCTION

The liver plays a crucial role in energy metabolism and thus hepatic dysregulation can contribute to the development of metabolic diseases [1]. Non-alcoholic fatty liver disease (NAFLD) is a pathological syndrome distinguished by excessive lipid droplets in liver cells, which are closely related to insulin resistance (IR), especially hepatic IR [2]. A high-fat diet (HFD) is known to increase lipid accumulation and impair β-oxidation in the liver and HFD-induced lipid accumulation can inhibit insulin degradation in the liver, therefore contributing to hyperinsulinemia [3]. Hyperinsulinemia increases the chance to precede prediabetes which is characterized by impaired glucose tolerance (140≤ glucose level ≤199 mg/dL in response to 70 g oral glucose) or impaired fasting glucose (IFG) (100≤ fasting plasma glucose ≤125 mg/dL) level in human [4]. Since prediabetes

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is one of the major risk factors, a therapeutic strategy to reduce liver fat accumulation is crucial for preventing the progression of type 2 diabetes mellitus (T2DM) and NAFLD [2].

As a non-therapeutic lifestyle-changing strategy, the beneficial effects of exercise on decreasing diabetes rates have been widely known. Endurance training can significantly reduce symptoms in prediabetic patients [5]. Accumulated data also demonstrate that regular exercise can protect against chronic diseases including T2DM and improve IR. Exercise is known to stimulate insulin sensitivity and alter lipid metabolism in the liver via lipid oxidation [6]. Previously, it was reported that regular exercise can reduce the hepatic fat content by 60% to 80%, resulting in improved insulin sensitivity in the liver [7]. Additionally, exercise can transiently decrease hepatic lipid levels by increasing fatty acid (FA) uptake and β-oxidation in muscles, and thus hepatic lipid storage can be reversed after prolonged exercise training (EX). Combined, previous studies suggest the beneficial effect of exercise on diacylglycerol (DAG) accumulation in the liver [3].

Additionally, besides exercise, nutritional interventions have been regarded as other non-therapeutic treatments for metabolic disorders [6]. Coffee, one of the most consumed beverages worldwide, has been introduced for its important source of antioxidants particularly due to the high amounts of phenolic compounds and caffeine [8]. Recently, green coffee (GC), an unroasted raw coffee bean, is being marketed as a popular dietary supplement for weight loss due to the hallmark that contains more chlorogenic acid (CGA) than any other roasted coffee [9]. GC contains seven distinctive hydroxycinnamic acid derivatives as CGA isomers, which constitute 10.4% of the total dry weight of GC products [10]. CGA is also a polyphenol and ester of caffeic and quinic acid which has been suggested to prevent ectopic lipid accumulation as well [11]. Previous *in vitro* studies presented that CGA can diminish lipid accumulation and CGA combined with caffeine can inhibit adipogenesis stronger than CGA or caffeine alone in 3T3-L1 cells [12,13]. In a current study, the role of CGA as a ligand to nuclear receptors that activate the metabolism of FAs has been proposed [14] and thus the authors propose that CGA could be a capable supplement to prevent obesity and dyslipidemia by lowering the total cholesterol (TC) synthesis and triacylglycerol (TG) levels in liver cells [15]. In animal studies, CGA has also been found to exert therapeutic effects on metabolic disorders, such as purifying extra FAs and increasing insulin sensitivity [16], helping to recover from metabolic disorders [17]. Together, previous data suggest the potential role of GC and/or CGA intake in preventing and/or treating metabolic disorders. Moreover, accumulated evidence that EX can exert metabolically beneficial effects also raises the possibility that combined treatments with EX and GC and/or CGA intake would be synergetic.

We have recently found that EX combined with GC or CGA can improve the symptoms and complications by regulating the glycogen synthesis mechanism [18]. However, there is still a lack of research evidence that EX and these nutritional treatments influence lipogenesis in prediabetic liver, which is crucial in NAFLD. Therefore, as a preclinical *in vivo* investigation, we first aimed to investigate the potential therapeutic effects of GC and CGA intake and EX on HFD-induced lipogenesis in the setting of prediabetes conditions using a mouse model. The effects of GC or CGA supplementation paired with EX were compared to the effects of a singular treatment to determine a possible synergetic effect on hepatic lipid metabolism in the prediabetes stage. In addition, the effects between GC and CGA intake on prediabetes conditions were compared to assess whether the effects of GC are mainly ascribed to CGA.

## METHODS

Detailed methods are available in the Supplementary Methods (Supplementary Table 1, Supplementary Fig. 1).

### Animals

All mice were treated according to the recommendations of the Animal Ethics Committee of the Royan Institute and the protocol was approved by the Ethics Committee of the Royan Institute. Male C57BL/6 mice were treated with a normal diet (ND; 10% fat, 20% protein, 70% carbohydrate) or HFD (60% fat, 20% protein, 20% carbohydrate) for 12 weeks as the first stage. After the first stage, HFD-treated mice were divided into six groups and applied the following treatments for additional 10 weeks: no treatment (pre-D), GC intake, CGA intake, EX, GC+EX, and CGA+EX. Mice were fed with the same diet until the end of the study.

## RESULTS

HFD during 12 weeks increased body weight (BW) in the pre-D group compared to the ND group (Table 1). This difference was further increased after additional 10 weeks (Supplementary Fig. 2A). All treatment groups, excluding GC, had a lower BW

**Table 1.** Differences in body weight, liver weight, liver to body weight ratio, and adipose tissue weight among groups

| Group | BW, g | | | LW, g | LW to BW ratio | Adipose tissue weight, g |
|---|---|---|---|---|---|---|
| | 1st week | 12th week | 23rd week | | | |
| ND | 13.3±0.8 | 28.2±0.9 | 30.8±0.9 | 3.88±0.73 | 0.13±0.02 | 0.50±0.08 |
| pre-D | 12.5±1.0 | 30.5±1.0[b] | 32.9±0.5[b] | 4.85±0.74 | 0.15±0.02 | 0.57±0.14[a] |
| GC | - | - | 31.5±1.1 | 3.79±0.48[c] | 0.12±0.01[c] | 0.50±0.12 |
| CGA | - | - | 31.0±0.9[d] | 3.68±0.44[c] | 0.11±0.01[c] | 0.52±0.15 |
| EX | - | - | 30.7±0.4[d] | 4.73±0.37 | 0.15±0.01 | 0.46±0.10 |
| GC+EX | - | - | 30.3±0.9[d] | 4.86±0.90 | 0.16±0.03 | 0.50±0.16 |
| CGA+EX | - | - | 30.5±1.1[d] | 4.59±0.80 | 0.10±0.00 | 0.47±0.18 |

Values are presented as mean±standard deviation.
BW, body weight; LW, liver weight; ND, normal diet; pre-D, no treatment; GC, green coffee; CGA, chlorogenic acid; EX, exercise training; GC+EX, green coffee and exercise training; CGA+EX, chlorogenic acid and exercise training.
[a]$P<0.05$ compared with ND in the same week, [b]$P<0.01$ compared with ND in the same week, [c]$P<0.05$ compared with pre-D in the same week, [d]$P<0.01$ compared with pre-D in the same week.

than the pre-D group, and the GC+EX group had the lowest BW. The pre-D group had a higher liver weight (LW) than the ND group, which was significantly lowered in the GC and CGA groups. These indicate that GC and CGA supplementation and EX could ameliorate a BW increase in response to HFD.

HFD consumption for the first 12 weeks increased fasting blood glucose (FBG) levels above the prediabetes threshold (100≤ IFG ≤125 mg/dL) (Supplementary Fig. 3). At the end of the second stage after additional 10 weeks, HFD mice (pre-D) had further increased FBG levels and area under the curve compared to the ND group (Fig. 1A, B and Supplementary Fig. 2B). Increased FBG levels in the pre-D group were accompanied by higher insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) index (Fig. 1A-D). Lipid profiles of the pre-D group also showed an increase in TC, TG, and low-density lipoprotein (LDL) levels compared to the ND group (Fig. 1E-G). Interestingly, high-density lipoprotein (HDL) levels were elevated in the pre-D group (Fig. 1H). Levels of alanine transaminase (ALT) and aspartate transaminase (AST), indicators of hepatic tissue damage, were also increased in the pre-D group compared to the ND group. However, these increases were alleviated by treatments (Fig. 1I and J). A significant reduction in FBG levels was found in all treatment groups compared to the pre-D group (Fig. 1A and B). In addition, all treatment groups showed a significant decrease in plasma insulin levels and HOMA-IR index compared to the pre-D group (Fig. 1C and D), demonstrating that all treatments mitigated HFD-induced prediabetes conditions in glucose profiles.

The lipid profiles showed a different pattern. For TC, CGA, EX, and GC+EX groups had a reduction compared to the pre-D group (Fig. 1E). TG levels were reduced in CGA, EX, and CGA+EX groups (Fig. 1F). LDL levels were lower in all treatment groups than in the pre-D group (Fig. 1G). As expected, HDL level in the EX-group was increased compared to the pre-D group. Notably, HDL levels were decreased in the GC, CGA, and GC+EX groups compared to the pre-D group (Fig. 1H). ALT and AST levels showed decreased levels in all treatment groups compared to the pre-D group (Fig. 1I and J). Furthermore, lipid accumulations were significantly higher in the livers from the pre-D group than from the ND group, and this increase was partially reduced in the livers from all treatment groups (Supplementary Fig. 4). These findings indicate that GC and CGA supplementation and EX can mitigate HFD-induced prediabetes conditions in circulating levels and fat accumulation in the liver. However, no synergistic effects of GC or CGA intake with EX were apparent. Additionally, the effects of GC and CGA intake on HFD-induced prediabetes conditions were similar, suggesting that the effects of GC would be primarily attributed to CGA effects in biochemical features.

The Gene Expression Omnibus (GEO) database was searched to identify candidate gene(s) possibly changed in response to HFD, GC treatment, and EX in the mouse liver. Based on analyzing data from GSE53131, 647, and 496 genes were identified to be up- and down-regulated, respectively, in response to HFD (Fig. 2A), while 256 and 160 genes were up- and down-regulated, respectively, by GC (Fig. 2B) (log fold change criteria >0.5 and false discovery rate <0.05). In addition, data from

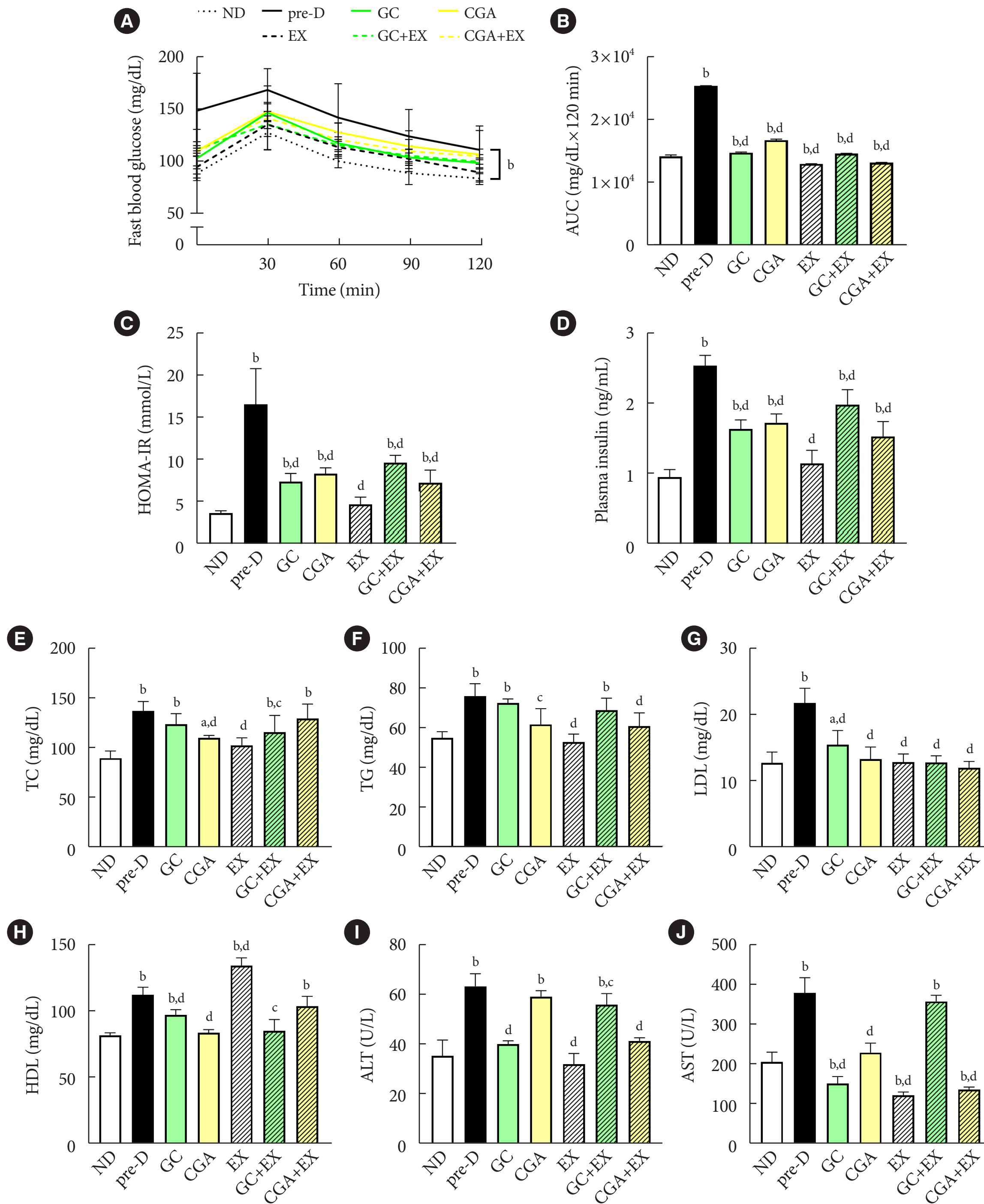


**Fig. 1.** Treatments mitigated high-fat diet (HFD)-induced prediabetic phenotypes. At the first stage of the study, mice were treated with two different diets for the first 12 weeks: a normal diet (ND, $n$=7) and HFD ($n$=42). Then, in the second stage, HFD mice were further divided into six groups ($n$=7/group): no treatment (pre-D), treated with green coffee (GC), chlorogenic acid (CGA), exercise training (EX), GC+EX, and CGA+EX. Treatments were applied for 10 weeks under the same diet. At the end of the second stage (22nd week), (A) glucose tolerance test, (B) the area under the curve (AUC), (C) the homeostatic model assessment for insulin resistance (HOMA-IR) index, (D) plasma insulin, (E) total cholesterol (TC), (F) triglyceride (TG), (G) low-density lipoprotein (LDL), (H) high-density lipoprotein (HDL), (I) alanine transaminase (ALT), and (J) aspartate transaminase (AST) levels were assessed. [a]$P$<0.05 vs. ND, [b]$P$<0.01 vs. ND, [c]$P$<0.05 vs. pre-D, [d]$P$<0.01 vs. pre-D.

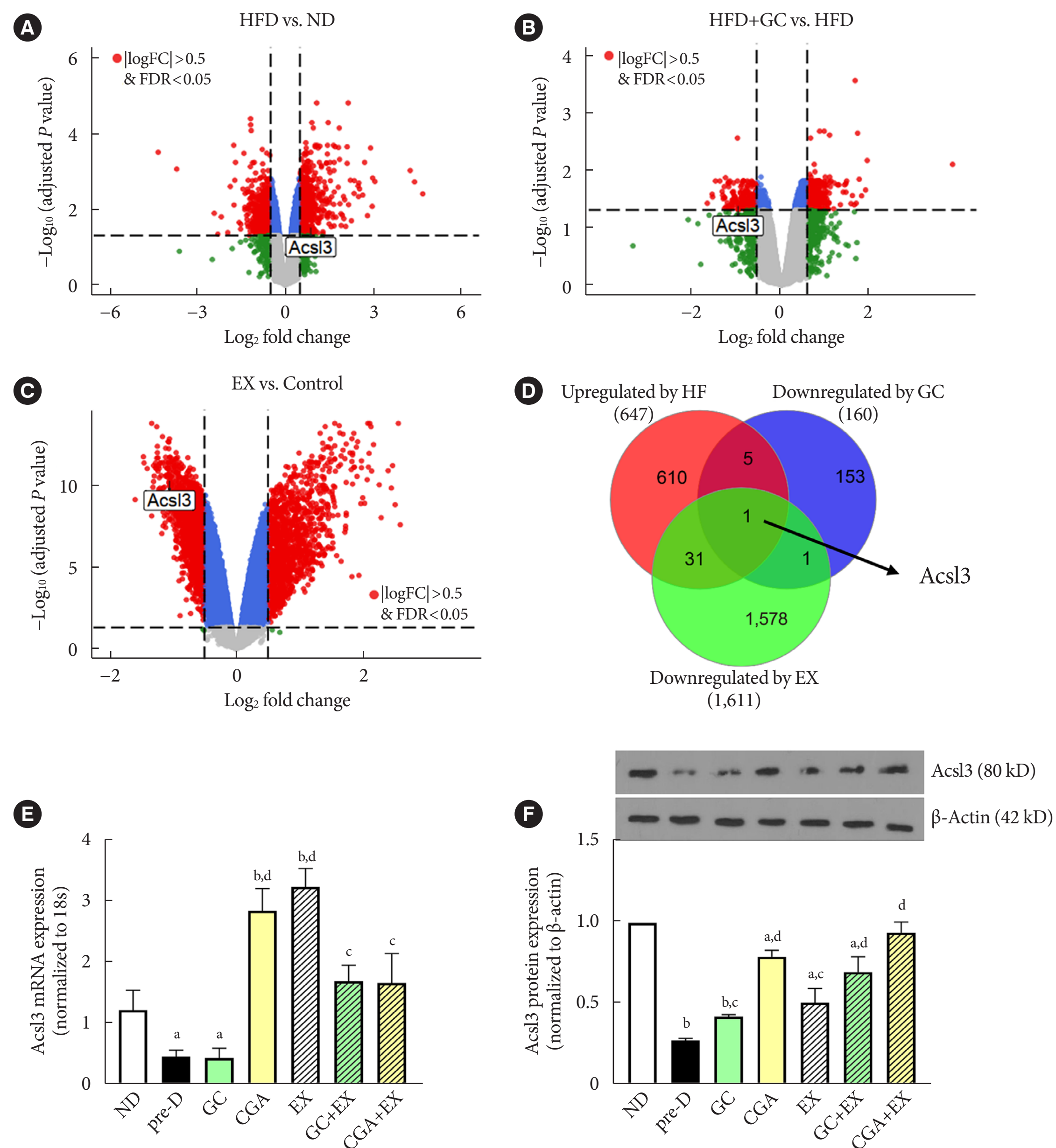


**Fig. 2.** Acyl-CoA synthetase long chain family member 3 (Acsl3) as a candidate gene is predicted to be differentially expressed with a high-fat diet (HFD), green coffee (GC), and/or exercise training (EX) in mouse livers and its responses to treatments. (A) Volcano plot showing differentially expressed genes with HFD compared to normal diet (ND) groups based on the data from GSE53131. (B) Volcano plot showing genes differentially expressed with GC intake in the setting of HFD based on the data from GSE53131. (C) Volcano plot showing differentially expressed genes with exercise compared to control groups based on the data from GSE104079. The red color represents the genes (|log fold change [FC]| >0.5 and false discovery rate [FDR] <0.5) significantly altered by each comparison. (D) The Venn diagram presents the distribution of significantly altered genes in three different comparisons. At the first stage of the study, mice were treated with two different diets for the first 12 weeks: a ND ($n$=7) and HFD ($n$=42). Then, in the second stage, HFD mice were further divided into six groups ($n$=7/group): no treatment (pre-D), treated with GC, chlorogenic acid (CGA), EX, GC+EX, and CGA+EX. Treatments were applied for 10 weeks under the same diet. At the end of the second stage (22nd week), Acsl3 gene (E) and protein (F) expression levels in the liver were measured using quantitative reverse transcription polymerase chain reaction and immunoblotting, respectively. 18s and β-actin were used to normalize gene and protein expression, respectively. [a]$P<0.05$ vs. ND, [b]$P<0.01$ vs. ND, [c]$P<0.05$ vs. pre-D, [d]$P<0.01$ vs. pre-D.

GSE104079 revealed that 1,351 and 1,611 genes were up- and down-regulated, respectively, by exercise (Fig. 2C). Acyl-CoA synthetase long chain family member 3 (*Acsl3*) was found to be a common gene among three different comparisons (Fig. 2D); up-regulation with HFD and down-regulation by GC and EX. Thus, this gene was chosen as a candidate gene, and assessed its gene and protein expression in this study. Oppositely to the anticipation by *in silico* analysis, *Acsl3* gene expression was reduced in the pre-D group compared to the ND group, while this reduction was reversed in all treatment groups (Fig. 2E). A similar pattern for the ACSL3 protein was found (Fig. 2F), suggesting that acyl-CoA production was decreased by HFD, while this reduction was mitigated by GC and CGA supplementation and EX. However, no synergetic effects of combined treatments were not obvious.

The expression of sterol regulatory element-binding protein 1C (*Srebp-1c*), a master gene in *de novo* lipogenesis (DNL), was elevated in the pre-D group compared to the ND group, while this elevation was attenuated in all treatment groups (Fig. 3A). Fatty acid synthase (*Fasn*) gene expression increased in the pre-D group compared to the ND group. However, no treatment mitigated this HFD-induced increase in *Fasn* gene expression (Fig. 3B). The expression of the acetyl-CoA carboxylase 1 (*Acc1*) gene, which encodes the first rate-limiting enzyme in DNL, was significantly increased in the pre-D group compared to the ND group. This increase was ameliorated in treatment groups, except for EX and CGA+EX groups (Fig. 3C). ACC1 protein expression was also higher in the pre-D group than in the ND group, however, treatment groups, excluding the GC group, had significantly reduced ACC1 protein expression compared to the pre-D group (Fig. 3D). These findings suggest that GC and CGA supplementation and EX can suppress HFD-induced activation of the DNL-related signals in the liver. Both gene and protein levels of glycerol-3-phosphate acyltransferase 1 (GPAT1) were significantly upregulated in the pre-D group compared to the ND group. These increases were alleviated by treatments with some exceptions (Fig. 3E and F), suggesting that HFD leads to increased hepatic TG production, which can be ameliorated by GC and CGA supplementation and EX. The effects of GC and CGA intake on DNL- and TG-producing signals in the setting of HFD were similar. This implies that the effects of GC are mainly attributed to CGA effects. GC or CGA intake combined with EX might not have synergetic effects.

The expression level of peroxisome proliferator-activated receptor alpha (*Ppar-α*), a ligand-activated transcription factor involved in β-oxidation, was elevated in the pre-D group compared to the ND group, while this elevation was diminished in CGA, GC+EX, and CGA+EX groups (Fig. 4A). *Acc2* gene expression was also upregulated in the pre-D group compared to the ND group, and the GC and CGA groups showed significantly reduced *Acc2* gene expression levels compared to the pre-D group (Fig. 4B). However, all treatments exerted no effects on ACC2 protein levels, although the pre-D group had a higher ACC2 protein level than the ND group (Fig. 4C). Similarly, although carnitine palmitoyl transferase I (*Cpt1*) gene expression was upregulated in the pre-D group compared to the ND group, treatments showed minimal effects on *Cpt1* gene expression (Fig. 4D). Interestingly, CPT1 protein expression showed a pattern opposite to *Cpt1* gene expression in that CPT1 protein expression level was lower in the pre-D group than in the ND group and higher in all treatment groups than in the pre-D group (Fig. 4E). These results suggest that β-oxidation might be reduced with HFD, while GC and CGA supplementation and EX could mitigate a HFD-induced decrease in β-oxidation. No difference between GC and CGA intake implies that the effects of GC are mainly due to CGA effects. Meanwhile, the synergistic effect of combined treatment of GC or CGA supplementation with EX was not evident for β-oxidation signals.

## DISCUSSION

HFD and a sedentary lifestyle are risk factors for T2DM and NAFLD, which are associated with increased mortality [4]. Hepatic dysfunction is known to contribute to the development of metabolic disorders, emphasizing the pivotal role of the liver in energy metabolism [19]. HFD increases lipid accumulation in the liver which can contribute to hyperinsulinemia and NAFLD [2,3]. ACSL3, a member of the ACSL family in lipid droplets, is known to play a role in DNL in the liver [20]. DNL begins with ACC and FASN producing FAs [21]. A previous study showed that GPAT1 has a role in mediating DAG production in the liver as a source of IR, and DAG formed during DNL influences cell lipid content [22]. HFD is known to activate SREBP-1c-mediated GPAT1 gene expression [23]. Another important regulator of FAs is PPAR-α, which is a transcriptional factor for β-oxidation and CPT1 in the liver; upon activation, PPAR-α promotes β-oxidation, releasing FAs and thereby increasing acetyl-CoA production from lipids stored

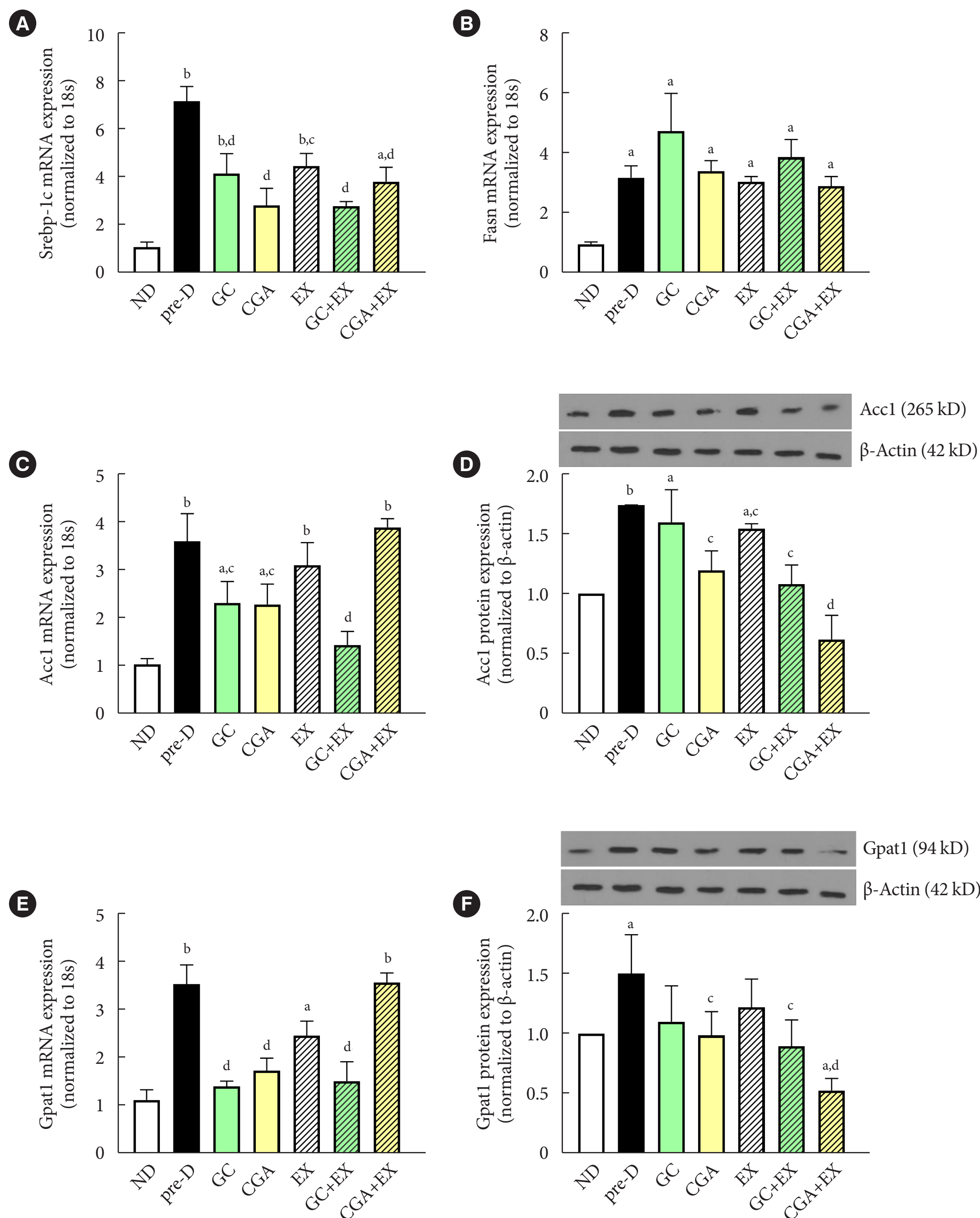


**Fig. 3.** Treatments attenuated high-fat diet (HFD)-induced increases in triglyceride production in the liver. At the first stage of the study, mice were treated with two different diets for the first 12 weeks: a normal diet (ND, $n=7$) and HFD ($n=42$). Then, in the second stage, HFD mice were further divided into six groups ($n=7$/group): no treatment (pre-D), treated with green coffee (GC), chlorogenic acid (CGA), exercise training (EX), GC+EX, and CGA+EX. Treatments were applied for 10 weeks under the same diet. At the end of the second stage (22nd week), (A) sterol regulatory element-binding protein-1c (Srebp-1c), (B) fatty acid synthase (Fasn), (C) acetyl-CoA carboxylase 1 (Acc1), and (E) glycerol-3-phosphate acyltransferase 1 (Gpat1) gene expression levels in the liver were measured using quantitative reverse transcription polymerase chain reaction. (D) ACC1 and (F) GPAT1 protein expression levels in the liver were assessed by immunoblotting. 18s and β-actin were used to normalize gene and protein expression, respectively. $^{a}P<0.05$ vs. ND, $^{b}P<0.01$ vs. ND, $^{c}P<0.05$ vs. pre-D, $^{d}P<0.01$ vs. pre-D.

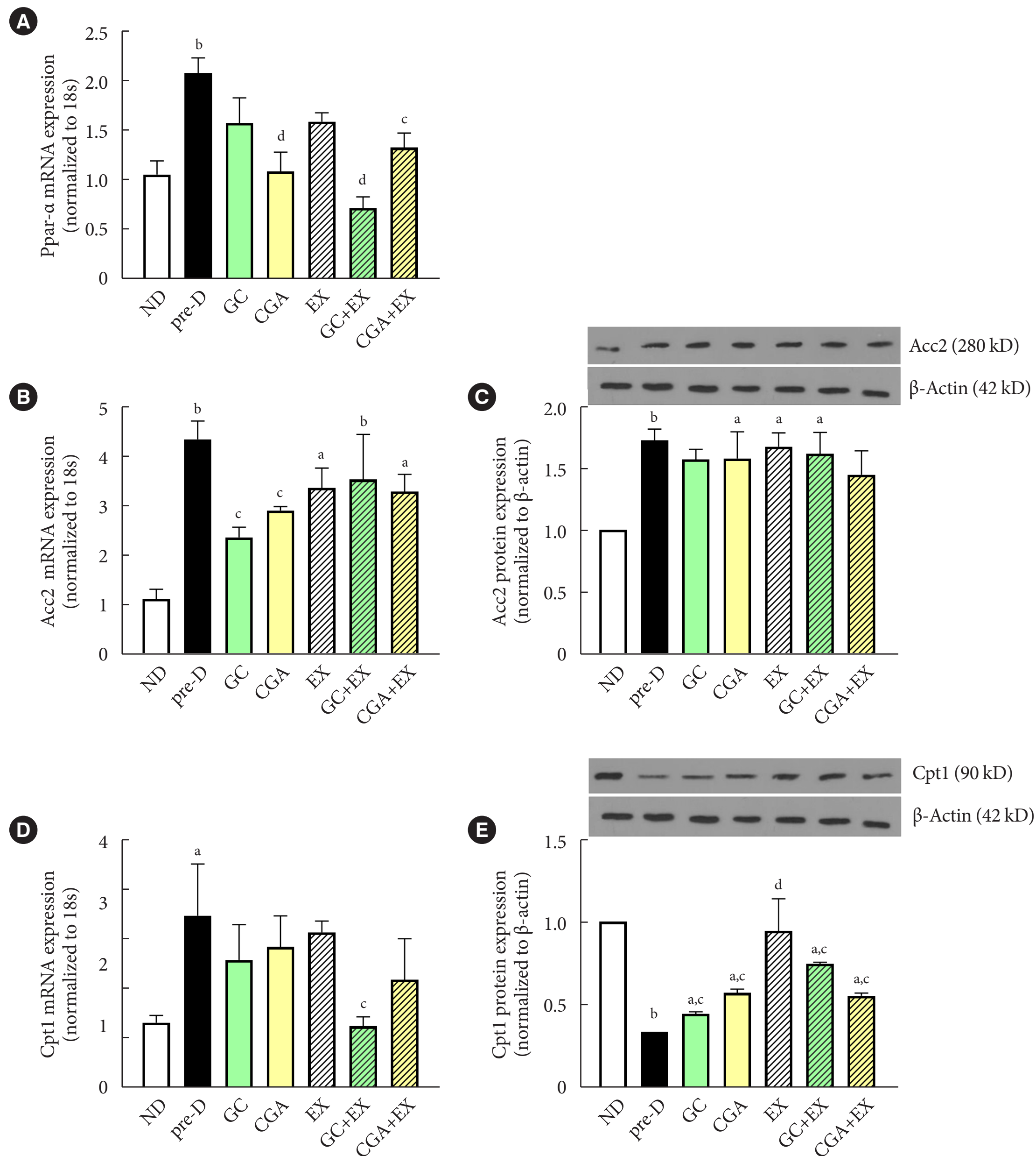


**Fig. 4.** Treatments mitigated high-fat diet (HFD)-induced decreases in β-oxidation in the liver. At the first stage of the study, mice were treated with two different diets for the first 12 weeks: a normal diet (ND, $n$=7) and HFD ($n$=42). Then, in the second stage, HFD mice were further divided into six groups ($n$=7/group): no treatment (pre-D), treated with green coffee (GC), chlorogenic acid (CGA), exercise training (EX), GC+EX, and CGA+EX. Treatments were applied for 10 weeks under the same diet. At the end of the second stage (22nd week), (A) peroxisome proliferator-activated receptor alpha (Ppar-α), (B) acetyl-CoA carboxylase 2 (Acc2), and (D) carnitine palmitoyl transferase I (Cpt1) gene expression levels in the liver were measured using quantitative reverse transcription polymerase chain reaction. (C) ACC2 and (E) CPT1 protein expression levels in the liver were assessed by immunoblotting. 18s and β-actin were used to normalize gene and protein expression, respectively. $^aP<0.05$ vs. ND, $^bP<0.01$ vs. ND, $^cP<0.05$ vs. pre-D, $^dP<0.01$ vs. pre-D.

in the liver [24]. Therefore, activating β-oxidation and/or deactivating DNL could be effective in treating prediabetes.

In this study, HFD increased BW, FBG, and insulin levels, indicating that HFD causes prediabetes even in young male

mice, while treatments demonstrated anti-HFD effects. As expected, EX diminished these increases. It is known that EX reduces insulin and FBG levels by elevating muscle glucose uptake and enhancing insulin sensitivity [18,25]. Consistently, EX shows the greatest effects on reducing FBG, HOMA-IR, and insulin levels among the treatments. As the prior studies suggested that GC or CGA consumption can influence lipid metabolism [9,17], this study provides further evidence supporting that GC and CGA can reduce lipid and glycemic complications under HFD consumption. One putative mechanism underlying GC and CGA effects could be the reduction in intestinal absorption of glucose via the CGA-associated inhibition of alpha-amylase, which ultimately can reduce the circulating insulin levels [26]. Therefore, further studies are warranted to elucidate this mechanism.

Lipid profiles were increased in the pre-D group compared to the ND group, while these increases were ameliorated by GC and CGA supplements and EX. LDL level was reduced by treatments up to the levels of the ND group, suggesting that GC and CGA supplements and EX can help reduce lipid profiles. These results could be associated with the effect of EX on proteinase subtilisin kexin 9 (PCSK9) modulation in that the inhibition of PCSK9 increases LDL absorption through its receptor [27]. Moreover, it was proposed that GC and CGA can ameliorate TC, TG, and LDL levels through the regulation of glucose transporter type 4 (GLUT4) in peripheral tissues, inhibition of intestinal α-amylase, increased hepatic glucokinase activity, inhibition of glucose 6-phosphatase, and increased transmission of glucose to adipose tissue [28], thus possibly lowering cholesterol levels. Measurements of lipid accumulation in the liver are also in line with the results of the circulating level, further supporting that HFD-associated increases in lipids could be partially prevented by GC and CGA supplements and/or EX. Notably, the pre-D group showed an increase in HDL levels. A previous study has also shown that HFD can increase HDL levels by increasing the transport rates and decreasing the fractional catabolic rates of HDL [29]. The EX group showed an increase in HDL levels similar to previous data that EX can increase lecithin–cholesterol acyltransferase, leading to increased cholesterol in HDL and thereby increasing circulating HDL [30]. Interestingly, GC and CGA supplementation significantly decreased HDL levels, even when combined with EX. A previous study has shown that CGA operates through the pro-atherogenic pathway of cholesterol metabolism [31], implying that GC or CGA could counteract the HDL-stimulating effect of exercise. Although lipid accumulation in the liver was increased in the pre-D group and diminished in all treatment groups, LW and LW to BW ratio were not exactly matched. It is likely that decreased LW and LW to BW ratio in CGA and GC, but not when combined with exercise (GC+EX and CGA+EX), might be associated with the increase in FAs synthesis due to GC caffeine or "athletic paradox" [32,33].

Given the relationship between lipid accumulation and AST and ALT levels via elevated reactive oxygen species (ROS) production [34], it can be speculated that an increase in ALT and AST levels in the pre-D group is associated with increased ROS accompanied by increased BW and adipose tissue weight [35] in the pre-D group. It can be further supported by the finding that the EX group had the lowest adiposity and AST and ALT levels. Previously, an antioxidant role of GC and CGA via their hydroxyl groups has been proposed [10]. The findings that increased AST and ATL levels with HFD were diminished by GC and CGA also support the antioxidant roles of GC and CGA.

*In silico* analysis of repository data revealed that *Acsl3* is a candidate gene that is possibly up-regulated by HFD and down-regulated with GC and EX in the mouse liver. ACSL3 is also known to play a critical role in lipid metabolism as ACSL enzymes are important for β-oxidation and lipid synthesis through FA activation [20]. In this study, our results are unexpectedly opposite in that both gene and protein expression of ACSL3 were significantly decreased by HFD and treatments partially reversed this HFD effect. This inconsistency could be due to several reasons, such as diverse metabolic disease states (diabetes vs. prediabetes), diet (fasting vs. HFD), and training intensity/duration. For example, for the database of GSE104079, array data from fasted control mice and fasted-EX mice were compared. To our knowledge, this is the first time to provide that decreased ACSL3 gene and protein expression were ameliorated by GC and CGA supplementation and EX in the mouse liver. Further supported by previous findings that an increase in FAs leads to a decrease in ACSL3 expression [36, 37], additional studies elucidating the mechanisms by which GC and CGA supplementation and EX activate ACSL3 are warranted.

IR plays a pivotal role in NALFD via DNL which is highly prevalent in individuals with T2DM [38]. In this study, HFD activated SREBP-1C, an initiator, and ACC1, a rate-limiting enzyme, which are known to facilitate malonyl-CoA that *FASN* produces palmitate, in the DNL process [23]. Similarly, the up-

regulation of *Srebp-1c*, *Acc1*, and *Fasn* was observed in the pre-D group and this was in line with an increase in BW and lipid profiles. These changes were mitigated with GC and CGA supplementation and EX. Notably, *Fasn* expression was not influenced by treatments despite an upregulation with HFD. *Fasn* expression tends to be even higher in the GC group than in the pre-D group, implying the caffeine effect in GC as discussed in a previous study that caffeine leads to an increase in *Fasn* gene expression [32]. Unfortunately, FASN protein expression was not assessed in this study.

GC and CGA supplementations reduced *Acc1* gene expression, while EX did not change *Acc1* gene expression in the setting of HFD. This would be associated with a reduction in entering FAs into the hepatic cells since long-term EX increases the consumption of FAs as the main energy source [39]. GPAT1 directs FAs to TG synthesis on the cytosolic surface of the mitochondrial external membrane and endoplasmic reticulum [23]. GC and CGA supplementation significantly reduced GPAT1 levels and this finding is in line with a reduction in TG levels.

PPAR-α is known to regulate the expression of key β-oxidation enzymes, including CPT-1 [40]. A significant increase in *Ppar-α* gene expression in response to HFD was found as previously observed [41] and this could be explained by FA production in hepatic cells by the FASN enzyme as it may act as a PPAR-α ligand [42]. ACC2 is located in the outer membrane of mitochondria and catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, an intermediate that inhibits the activity of CPT1 [43]. Thus, the inhibition of ACC2 results in enhanced oxidation of lipids [44]. In this study, HFD enhanced ACC2 expression at both gene and protein levels, while treatments, particularly EX, showed minimal effects on the changes in ACC2. Similarly, this would be associated with increased FAs usage during exercise. Notably, CPT1 gene and protein expression patterns were opposite, raising the potential mechanisms of mRNA half-life, stability of mRNA, and post-tran-

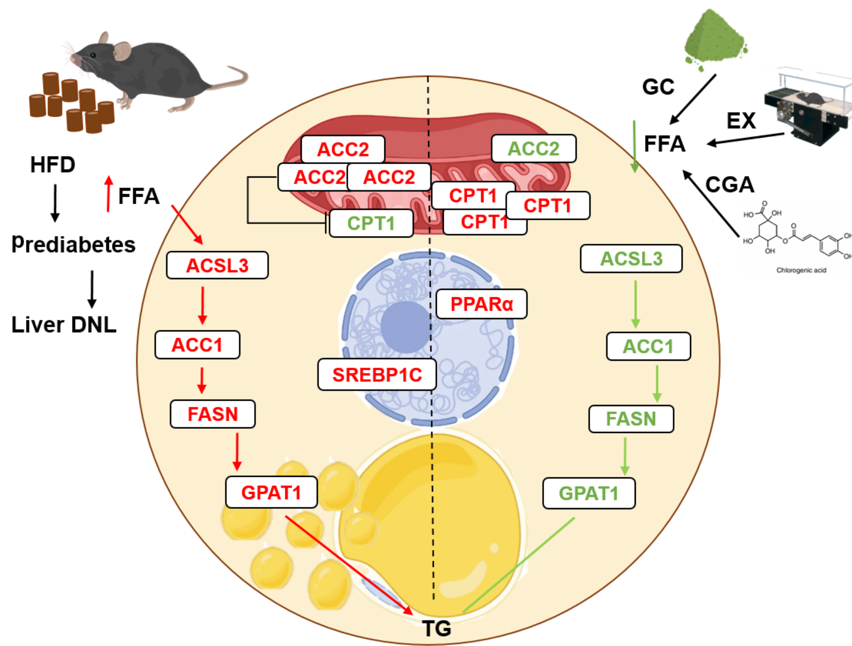


**Fig. 5.** Graphical abstract. A prediabetic hepatic cell representing *de novo* lipogenesis (DNL) (left) associated with a high-fat diet (HFD) and the preventive effects of treatments (right). HFD elevates plasma free fatty acids (FFAs) which enter hepatic cells and contribute to the activation of acyl-CoA synthetase (ACSL) known to convert fatty acids (FAs) into fatty acyl-CoAs. Acyl-CoAs then proceed with *de novo* lipogenesis (acetyl-CoA carboxylase 1 [ACC1]) and/or β-oxidation inhibition (ACC2). Consequently, with the involvement of fatty acid synthase (Fasn) and glycerol-3-phosphate acyltransferase 1 (GPAT1), triglyceride (TG) can be formed. In this study, lipogenic molecules were found to increase with HFD (red color) which can lead to an increase in lipid droplets inside the cell owing to increased TG production (left). However, exercise training (EX), green coffee (GC), and chlorogenic acid (CGA) treatments mitigated HFD-induced changes in the lipogenic molecules (green color). Thus, β-oxidation via carnitine palmitoyl transferase I (CPT1) can be increased, and FA levels and the lipid droplet size inside the hepatic cell can be decreased.

scriptional regulation [45,46], although this study does not provide supporting evidence.

We previously found that exercise combined with GC or CGA was effective in the production of glucose and glucose accumulation by controlling the genes involved in glucose metabolism [18]. However, in the present study, CG or CGA supplementation paired with EX exerts no synergistic effects on HFD-induced lipid complications compared to a single treatment. It can be speculated that the maximal benefit was achieved by a single treatment; therefore, there was no capacity to be improved further even with additional stimulation (denoted as "the ceiling effect"). Otherwise, the antagonistic interactions of two independent treatments together could be exerted. Despite improved glucose and lipid metabolism in the treatment groups compared to the pre-D group, the treatment groups still had higher or lower levels than the ND group. This emphasizes that eating less fat is the most important strategy in preventing glycemic and lipid complications. In addition, the GC group had similar results to the CGA group, and the CGA group tended to have better effects than GC in most cases, demonstrating that the beneficial effects of GC might be mainly due to CGA.

Some of the other health benefits from CGA supplementation with exercise have been proposed earlier, such as anti-fatigue activity by alleviating oxidative stress and improving muscle structure [47]. CGA is also proposed to improve the rate of strength recovery after exercise [48] and exert positive effects on skeletal muscle mitochondria as a new mechanism to induce an increase in muscle strength and performance [49]. Therefore, further studies examining the effect of GC and CGA supplementation with or without EX on physical function focusing on muscle quality and function will be interesting.

In brief (Fig. 5), GC and CGA supplementation and EX reversed or ameliorated HFD-induced prediabetes along with the adverse alterations in blood glucose profiles, lipid profiles and accumulation, and DNL- and β oxidation-associated molecules in the mouse liver. The exercise group showed the greatest beneficial effect; however, GC or CGA supplementation paired with EX did not exert a synergistic effect in most cases. The CGA group tended to have similar or better results than the GC group, suggesting that CGA can be a single supplement to help glucose and lipid hemostasis in the liver at the prediabetes stage. Together, these preclinical findings suggest that regular exercise is an effective non-therapeutic strategy to prevent prediabetes and CGA supplementation is recommended to partially mimic the beneficial effects of exercise on prediabetes conditions.

## SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at https://doi.org/10.4093/dmj.2022.0265.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## AUTHOR CONTRIBUTIONS

Conception or design: all authors.
Acquisition, analysis, or interpretation of data: S.S., S.M.M., S.K.K.
Drafting the work or revising: S.S., S.M.M., S.K.K.
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## SUPPLEMENTARY METHODS

### Animals

Forty-nine male C57BL/6 mice (Royan Institute, Isfahan, Iran) were purchased at 4 weeks of age (12 to 14 g of body weight). Mice were housed in the same room for the entire experimental period under the standard conditions (non-barrier) with a 12-hour light-dark cycle (7:00 AM to 7:00 PM) in a controlled temperature (23°C±1°C) and humidity (50% to 60%). All mice had *ad libitum* access to water and food. Body weight was recorded and the cage location was randomly changed weekly. All mice were treated as planned and had no health issues.

### Diet

After one habitation week upon arrival, mice were randomly divided into two groups: normal diet (10% fat, 20% protein, 70% carbohydrate; $n$=7) and high-fat diet (HFD; 60% fat, 20% protein, 20% carbohydrate; $n$=42) (Faradamzarin, Isfahan, Iran). These diets were applied for 12 weeks as the first stage of the study. After the first stage, HFD-treated mice were further randomly divided into six groups ($n$=7/group): (1) no treatment (pre-D), (2) green coffee (GC) intake, (3) chlorogenic acid (CGA) intake, (4) exercise training (EX), (5) GC+EX, and (6) CGA+EX. The corresponding treatments were applied for additional 10 weeks under the same diet as the first stage. The sample size ($n$=7/group) was determined using G*Power software for 0.75 of the effect size at a 5% significance level with 90% power [1].

GC tablets (Bonyan Salamat Kasra, Tehran, Iran) contain natural products; in particular, 400 mg of standardized GC bean extract powder containing 2% caffeine and 50% CGA [2]. The analysis of CGA in GC bean extract was performed using a high-performance liquid chromatography-diode array detector gradient system (Agilent 1090 series, Agilent, Santa Clara, CA, USA) (Supplementary Fig. 1). GC (200 mg/kg in 200 μL) or CGA (100 mg/kg in 200 μL) was administered three times per week as a gavage supplement (Sigma-Aldrich, St. Louis, MO, USA) dissolved in water, as previously described [3].

At the end of the second stage, the animals were fasted overnight (12-hour) and were anesthetized by intraperitoneal injection of a cocktail of 10% ketamine (50 mg/kg) and 2% xylazine (10 mg/kg). Blood was taken from the right ventricle, collected in tubes containing ethylene-diamine-tetraacetic acid (EDTA), centrifuged at 2,000 g for 10 minutes to obtain serum, and immediately stored at −80°C. Then, mice were euthanized by cervical dislocation. Liver tissues were immediately obtained and stored at −80°C for further analyses.

### Exercise training protocol

The exercise was performed on a rodent treadmill (MazeRouter, Tabriz, Iran) at a moderate intensity [2] for 10 weeks during the second stage of the study. Mice began to run on the treadmill at 17 m/min in the first 2 weeks. Then, the running speed was gradually increased every 2 weeks to reach 23 m/min in the final 2 weeks of the training period. Mice ran for 45 min/day and 5 day/week. Each exercise session was composed of 3 minutes of warm-up, 40 minutes of running, and 2 minutes of recovery. Not exercising mice were also placed on the treadmill but were not committed to running. The exercise was performed between 10:00 and 11:00 AM.

### Biochemical analyses

Fasting blood glucose and glucose tolerance tests (GTT) were performed both at the end of the first stage (12th week) and the second stage (22nd week) using a tail prick and glucometer (Alpha TRAK glucometer, Zoetisus, Parsippany-Troy Hills, NJ, USA). For GTT, 6-hour fasted mice were administered 200 μL glucose water solution by nasogastric feeding. Blood glucose levels were measured at 0, 30, 60, 90, and 120 minutes after feeding. Plasma insulin levels were determined using an Ultra-Sensitive Mouse Insulin enzyme-linked immunosorbent assay (ELISA) Kit (80-INSMS-E01, ALPCO, Salem, NH, USA), following the manufacturer's instructions. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using mouse AST (CSB-E12649m) and ALT (MBS016898) ELISA kits (Abcam, Cambridge, UK), respectively. High-density lipoprotein and low-density lipoprotein levels were determined using a kit (Cat. K613-100, Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's protocol. All ELISA assays were performed using an NS-100 Nano Scan Microplate Reader (Hercuvan Lab Systems, Cambridge, UK).

### *In silico* data analysis

To identify candidate genes regulated by HFD, GC, and exercise in the mouse liver, publicly published data from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) were searched and analyzed. The keywords GC, liver, HFD, and endurance training were used to obtain the qualified data. As a result, two studies with accession numbers

GSE53131 and GSE104079 were chosen for further analyses. Raw data from these studies were downloaded, and the initial preprocessors, including background correction, normalization, and data transfer in a logarithmic mode based on 2, were performed using affy and limma packages. The result of the expression matrix was used in the analysis, and information about the probes used in both studies was evaluated to identify candidate genes. Microarray data analysis was performed using the R software version 4.1 (R Foundation for Statistical Computing, Vienna, Austria). The linear model method was used to identify microarray data that differed between groups, and a false discovery rate of $<0.05$ was considered significant.

**RNA isolation and gene expression analysis**

RNA was isolated from frozen liver samples using the TRIzol protocol (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and purity of the extracted RNA were assessed using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). RNA (1,000 ng) with an integrity number ≥7.5 were transcribed into cDNA using a biotech rabbit cDNA Synthesis Kit (biotechrabbit GmbH, Berlin, Germany). Quantitative reverse transcription polymerase chain reaction was performed with Real QPlus 2x Master Mix Green with low ROX (Amplicon, Brighton, UK) using Step One Plus (Applied Biosystems, Waltham, MA, USA). The primers are listed in Supplementary Table 1. Relative gene expression was calculated using the ΔΔCt method.

**Protein extraction and immunoblotting analysis**

Frozen livers were lysed using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts (30 μg) of total protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. After blocking with 10% skim milk, the membranes were incubated with primary antibodies for 2 hours at room temperature (23°C±1°C). The primary antibodies used were as follows: acyl-CoA synthetase 3 (ACSL3; 1:1,000, sc-166374), acetyl-CoA carboxylase 1 (ACC1; 1:1,000, sc-137104), acetyl-CoA carboxylase 2 (ACC2; 1:1,000, sc-390344), glycerol-3-phosphate acyltransferase 1 (GPAT1; 1:1,000, sc-398135), carnitine palmitoyl transferase 1 (CPT1; 1:1,000, sc-514555), and β-actin (1:1,000, sc-47778) (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then incubated for 1 hour at room temperature with the following secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; 1:5,000, P0447) (Agilent) and HRP-conjugated goat anti-rabbit IgG (1:16,000, sc-2301) (Santa Cruz Biotechnology). The band intensity was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Oil red O staining**

The frozen livers were sectioned and washed with phosphate buffer saline, then stained with a hematoxylin solution. As previously described [4], the sections were stained with oil red O (ORO) staining solution after incubation in 60% isopropanol for 10 minutes. Finally, the sections were washed with running water and were observed under am Olympus BX40 microscope (Olympus, Tokyo, Japan). ORO-stained sections were analyzed to quantify lipid content using ImageJ software [5].

**Statistical analysis**

Data are presented as the mean±standard deviation. Data from the second stage of this study were analyzed using one-way analysis of variance (ANOVA) to determine the statistical differences among groups. All statistical analyses were performed using SigmaPlot statistical software (version 12.5; https://sigmaplot.com/sigmaplot-ng) with Tukey *post hoc* test. The significance level was set at $P<0.05$.

**Ethics statements**

The animal study protocol was approved by the Ethics Committee of the Royan Institute (ethics code: IR. ACECR. ROYAN. REC. 1399.075). All animals were treated according to the recommendations of the Animal Ethics Committee of the Royan Institute.

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