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The Mechanisms of Neochlorogenic Acid (3-Caffeoylquinic Acid) in Improving glucose and Lipid Metabolism in Rats with Insulin Resistance Induced by A High Fat-High Fructose Diet

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Abstract

This study investigated the effects and the mechanism of actions of neochlorogenic acid (3-caffeoylquinic acid; 3-CQA) on glucose and lipid metabolism in rats fed a high fat-high fructose diet (HFFD). Male rats were fed HFFD (40 % lard and 20 % fructose) for 16 weeks. At the 10th week, the HFFD rats were split into 3 groups: HFFD receiving distilled water (HFFD control group), HFFD receiving 3-CQA 50 mg/kg and HFFD receiving metformin 200 mg/kg once daily for a further 6 weeks. At the end of treatment, fasting blood glucose (FBG), oral glucose-tolerance test (OGTT), lipid profile, insulin, leptin, adiponectin, markers of oxidative stress, and hepatic triglyceride content were measured. Liver, adipose tissue, and skeletal muscle were collected for histological, gene and protein examinations. Compared to the HFFD control group, the 3-CQA group exhibited significantly reduced FBG, insulin and leptin levels, and improved OGTT. Serum adiponectin increased and lipid profiles were normalized. Hepatic triglyceride was reduced with a decrease in lipid droplets in liver histological sections. Levels of serum SOD and CAT activity, and MDA were reversed by 3-CQA treatment. Moreover, 3-CQA significantly reduced the expression of adipocyte pro-inflammatory cytokine genes (*MCP-1*, *TNF-α*, and *IL-6*), hepatic lipogenic genes (*SREBP1c*, *FAS*, and *GPAT*), and hepatic gluconeogenic genes (*PEPCK* and *G6Pase*). Additionally, 3-CQA increased expression of muscle *GLUT4* gene, and of GLUT4 protein with increased p-AKT and p-AMPK in skeletal muscle. In conclusion, 3-CQA improves glucose and lipid metabolism plausibly by decreasing oxidative stress and inflammation-induced insulin resistance, downregulating the expression of lipogenic and gluconeogenic genes, and enhancing insulin signaling in HFFD-induced insulin-resistant rats.

Keywords: Neochlorogenic acid, 3-Caffeoylquinic acid, Insulin resistance, Gluconeogenic genes, Glucose uptake, Lipogenic genes, AKT-AMPK pathway

Introduction

Obesity is the main risk factor for insulin resistance [1], and insulin resistance induces the development of metabolic disorders, including type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), and dyslipidemia [2]. Insulin resistance is characterized by a diminished response to insulin stimulation of target tissues, such as the liver, muscle, and adipose tissue, resulting in decreased glucose uptake in muscle cells and adipocytes, impaired hepatic glycogen storage, increased hepatic glucose production and adipocyte lipolysis [3]. Diets high in fat or fructose promote insulin resistance and elevate lipid influx to the liver together with increased hepatic *de novo* lipogenesis (DNL) [3,4]. Besides hyperinsulinemia, consumption of a diet high in fructose can increase mRNA expression of nuclear transcription factor sterol regulatory element binding protein 1c (SREBP1c) which plays a key role in the regulation of DNL. A high-fructose diet can also lead to reduced activity of peroxisome proliferator-activated receptor α (PPARα), resulting in the promotion of hepatic fat accumulation [5]. PPARα promotes transcription of genes involved in fatty-acid oxidation, resulting in increased mitochondrial and peroxisomal β-oxidation, and thereby reducing hepatic lipid levels [6]. The decreased lipogenesis and increased lipid oxidation brought about by effects of these key transcription factors may result in reduced fat accumulation in the liver and lipid levels in serum [7]. Likewise, glucose metabolism under insulin resistance, the gene expression of 2 rate-limiting enzymes involved in hepatic gluconeogenesis,

phosphoenolpyruvate carboxylase (PEPCK) and glucose 6-phosphatase (G6Pase) increase and promote hepatic glucose production [8].

In obesity, expanded adipose tissue secretes several pro-inflammatory molecules, including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) which may have local effects inducing insulin resistance in white adipose tissue (WAT) and also systemic effects on other organs. The secretion of other adipokines, such as adiponectin and leptin, from expanded adipose tissue are also changed. Adiponectin and leptin have an important role in regulating energy metabolism and body weight [9]. Glucose transporter-4 (GLUT4) mediates insulin action in uptake of glucose into adipose tissue and skeletal muscles. Particularly in skeletal muscle, GLUT4 is the most abundant glucose transporter accounting for 80 % of insulin-stimulated glucose transport [10]. Therefore, the control of glucose uptake by GLUT4 in skeletal muscle is considered to be a potential target for maintenance of glucose homeostasis and also for the treatment of T2DM [11].

Chlorogenic acids (CGAs) are derived from esterification of cinnamic acids, including caffeic, ferulic, and p-coumaric acids with quinic acid, and represent an abundant group of plant polyphenols present in the human diet. CGAs have different subgroups that include caffeoylquinic, p-coumaroylquinic, and feruloyquinic acids [12]. Caffeoylquinic acids (CQAs), esters of caffeic acid and quinic acid, are among the important polyphenols found in plant families Asteraceae and Lamiaceae [13], and are ones of the main components of coffee [14]. 3-caffeoylquinic acid (3-CQA or neochlorogenic acid) are present in several plants, such as Coffea spp., tomato, artichoke, potato, apples, and fresh stone fruits (peaches and plums) [13,14]. 5-CQA, also called chlorogenic acid, is the most abundant isomer among CQA derivatives (3-, 4-, and 5-CQA) with many beneficial effects on human health [13,15]. CQAs possess a wide range of pharmacological activities, including antioxidant, antibacterial, antiviral, anti-Alzheimer, and neuroprotective activities [15]. Furthermore, *in vitro*, CQAs, including 3-CQA has α-glucosidase and DPP-4 inhibitory activities [16]. Based on an experiment performed by Wu *et al*., 3-CQA was reported to suppress lipid accumulation by decreasing lipid synthesis and increasing lipid oxidation in HepG2 cells [17], indicating a potential effect of 3-CQA on hepatic lipid accumulation. However, still there is no evidence to demonstrate beneficial effects of 3-CQA in improving glucose and lipid metabolism, and in alleviating insulin resistance-associated disorders in rats fed a high fat-high fructose diet (HFFD). Therefore, the aims of this study were to investigate the potential effects of 3-CQA on glucose and lipid metabolism, and on insulin-resistant conditions, particularly its underlying molecular mechanisms, in HFFD-fed rats.

Materials and methods

Chemicals

Neochlorogenic acid (3-caffeoylquinic acid; 3-CQA with a purity of ≥ 98 % and molecular weight of 354.31) was obtained from Simagchem Corporation (Xiamen, Fujian, China). Thiopental was obtained from Unique Pharmaceutical Laboratories (A Div. of J.B. Chemicals & Pharmaceuticals Ltd.) (Ankleshwar, India) and metformin was from the Siam Pharmaceutical Company Ltd. (Bangkok, Thailand).

Animal and experimental protocols

Male Sprague-Dawley rats (160 - 180 g) were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. The animals were housed under a regulated room temperature regime (25 ± 2 °C) with a 12 h dark-light cycle at the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. All animal procedures were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kean, Thailand (IACUC-KKU-82/2562).

After a week of acclimatization, the rats were divided into 2 major groups. One group was fed a normal diet and regular drinking water throughout the experiment (n = 6, normal diet control group). The second group was fed a HFFD and 10 % fructose in drinking water for a period of 10 weeks. After 10 weeks of HFFD feeding, the HFFD rats were assigned to 3 groups (n = 6 each) as follows; HFFD rats receiving distilled water (HFFD control group), HFFD rats receiving 3-CQA (50 mg/kg BW/day), and HFFD rats receiving metformin (MET, 200 mg/kg BW/day as a positive control group) once daily by oral gavage for a further 6 weeks with continued HFFD feeding.

Normal rat diet (3.04 kcal/g) (CP Mice Feed, Samut Prakan Province, Thailand) was composed of 24 % protein, 4.5 % fat and 47 % carbohydrate. The HFFD (5.16 kcal/g) was composed of 40 % fat (lard), 20 % fructose, and 40 % normal rat diet. The 3-CQA and metformin were dissolved in distilled water and administered orally daily. Weekly body weight and daily food intake were recorded throughout the study period.

The experiment ended after 16 weeks. At this time, an oral glucose-tolerance test was performed in overnight-fasted animals, and blood samples were collected from the lateral tail vein to measure serum lipid, insulin, leptin and adiponectin. Then, the animals were sacrificed by intraperitoneal injection of thiopental sodium (80 mg/kg BW), and blood was collected from the abdominal vein to measure oxidative stress markers. Liver, visceral adipose tissues, and gastrocnemius muscle were rapidly excised and collected for further analysis as described below. The organ weights were normalized to the body weight.

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed after 10 - 12 h of fasting. Rats were orally given glucose (2 g/kg), followed by taking blood samples from the tail vein at 0, 30, 90 and 120 min. Blood glucose levels were measured using an Accu-Chek Performa glucose meter (Roche Diagnostics, Mannheim, Germany). An area under the curve (AUC) of blood glucose was calculated using a trapezoidal rule.

Biochemical analysis

Fasting serum insulin, leptin, and adiponectin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) kits (EMD Millipore, Billerica, MA, USA). Homeostasis model assessment of insulin resistance (HOMA-IR), an index of insulin resistance, was calculated using the following formula: Fasting glucose (mmol/L) x fasting insulin (µIU/mL)/22.5 [18].

The serum triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured using an enzymatic colorimetric method (Wako, Osaka, Japan). Serum free fatty acid (FFA) was also measured using an enzymatic colorimetric method (Abcam, Cambridge, MA, USA).

Liver TG concentrations were determined as previously described method [19]. Briefly, 50 mg of liver tissue were homogenized and extracted with 1 mL of isopropanol. After centrifugation, TG levels in the supernatant were measured using an enzymatic colorimetric method (Wako, Osaka, Japan).

The concentrations of serum malondialdehyde (MDA) were measured as thiobarbituric acid reactive substances (TBARS) as described previously [20]. 1, 1, 3, 3 tetraethoxypropane (TEP) was used as the standard. The MDA concentrations were expressed as µM.

Catalase (CAT) activity in serum was determined using a colorimetric assay based on the reaction of undecomposed hydrogen peroxide with ammonium molybdate to produce a yellowish color following a previously described method [21]. CAT activity was expressed as U/mL.

SOD activity in serum was determined based on the inhibition of nitroblue tetrazolium (NBT) reduction using the method of Sun *et al*. [22]. The enzymatic activity is presented in units of SOD activity (U/mL), where 1 unit of SOD is defined as the amount of enzyme required to cause 50 % inhibition of the reduction rate of nitroblue tetrazolium [23].

Histological analysis

Liver and epididymal fat pad were fixed in 10 % neutral buffered formalin, embedded in paraffin, and processed for staining with hematoxylin and eosin (H&E). Histological changes were observed using a light microscope (Carl Zeiss Microscopy, Jena, Germany). Sizes of adipocytes were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical study

Sections (4 µm) of gastrocnemius muscle were deparaffinized and rehydrated. After antigen retrieval, the sections were incubated in 0.3 % H_2O_2/methanol for 30 min. Then, the sections were blocked with 10 % normal goat serum and incubated with rabbit anti-GLUT4 antibody (Abcam, Cambridge, MA, USA). After that, the sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (Abcam, Cambridge, MA, USA). Then, the sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Finally, the sections were incubated with 3,3′-diaminobenzidine (DAB). After DAB staining, the sections were dehydrated again and mounted. Immunostaining was visualized with a light microscope (magnification 200×) (Nikon, NY, USA). The staining intensity of GLUT4 expression was measured using ImageJ Fiji software as previously described [24].

RNA isolation and real-time quantitative PCR

Total RNA was extracted from the liver and gastrocnemius muscle using TRIzol reagent (Invitrogen, San Diego, CA, USA). The cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad, CA, USA) according to the manufacturer's protocol. Real-time quantitative PCR was performed using CAPITAL™ QPCR Green Master Mix (Biotechrabbit, Berlin, Germany) on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Warrington, UK). Relative expression of the gene of interest was calculated using the $2^{-\Delta\Delta Ct}$ method. β-actin was used as an internal control. The primers are listed in **Table 1**.

Western blot analysis

Western blot analysis was performed as previously described [25]. Total protein (20 μg) of the gastrocnemius muscle were separated by 12 % SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), followed by blocking with 5 % non-fat powdered milk in Tris-buffered saline/Tween-20. The membranes were incubated with all target primary antibodies: Anti-phospho-AKT Ser473 (p-AKT), anti-AKT, anti-phospho-AMPKα Thr172 (p-AMPK), and anti-AMPK (EMD Millipore, Billerica, MA, USA), and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Protein bands were detected using Clarity™ Western ECL substrate (Bio-Rad, CA, USA). The images were obtained with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and band intensities were quantified using Gel-Pro Analyzer version 3.1 software (Media Cybernetics, Inc., Rockville, MD, USA). All relative target proteins were normalized to β-actin.

Statistical analysis

All results are shown as the means ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test for multiple comparisons analysis (SigmaStat Software, CA, USA). The acceptable value of significance was set at $p < 0.05$.

Table 1 The sequences of primers used for real-time quantitative PCR.

Genes	GenBank accession number	Primer sequences	Amplicon size (bp)
GLUT4	NM_012751.1	F: GAAACCAGAGGCCGGACATT R: ACCATTTTGCCCCTCAGTCA	136
PEPCK	NM_198780.3	F: GGGGGTGTTTACTGGGAAGG R: CGGTTCCTCATCCTGTGGTC	99
G6Pase	NM_013098.2	F: CGTCACCTGTGAGACTGGAC R: ACGACATTCAAGCACCGGAA	144
SREBP1c	NM_001276707.1	F: CCGAGGTGTGCGAAATGG R: TTGATGAGCTGAAGCATGTCTTC	64
FAS	NM_017332.1	F: TCGACCTGCTGACGTCTATG R: TCTTCCCAGGACAAACCAAC	196
GPAT	NM_017274.1	F: CCACATCAAGGATACAGCTCAT R: CATTCGTGTGTTTACATCGGC	145
PPARα	NM_013196.1	F: TAATTTGCTGTGGAGATCGGC R: TTGAAGGAGTTTTGGGAAGAGAA	140
CPT1	NM_031559.2	F: CAGCTCGCACATTACAAGGA R: TGCACAAAGTTGCAGGACTC	128
ACOX1	NM_017340.2	F: CTGATGAAATACGCCCAGGT R: GGTCCCATACGTCAGCTTGT	75
MCP-1	NM_031530.1	F: TGTCTCAGCCAGATGCAGTTAAT R: CCGACTCATTGGGATCATCTT	77
TNF-α	NM_012675.3	F: GTAGCCCACGTCGTAGCAAAC R: ACCACCAGTTGGTTGTCTTTGA	113
IL-6	NM_012589.2	F: GACTTCCAGCCAGTTGCCTT R: GCAGTGGCTGTCAACAACAT	50
β-actin	NM_031144.3	F: GGAGATTACTGCCCTGGCTCCTA R: ACTCATCGTACTCCTGCTTGCTG	150

F: Forward primer; R: Reverse primer. GLUT4, Glucose transporter-4; PEPCK, Phosphoenolpyruvate carboxykinase; G6Pase, Glucose 6-phosphatase; SREBP1c: Sterol regulatory element binding protein 1c, FAS: Fatty acid synthase, GPAT: Glycerol-3-phosphate acyltransferase, PPARα: Peroxisome proliferator activated receptor α, CPT1: Carnitine palmitoyl transferase 1, ACOX1: Acyl- CoA oxidase 1; MCP-1, Monocyte chemoattractant protein-1; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6.

Results and discussion

Results

Table 2 Effects of 3-CQA administered for 6 weeks on metabolic parameters.

Parameters	Groups			
	ND	**HFFD**	**HFFD + 3-CQA**	**HFFD + MET**
Body weight gain (%)	14.76 ± 1.14	31.60 ± 2.13*	21.08 ± 2.30$^{\#}$	20.24 ± 2.54$^{\#}$
Food intake (g/rat/day)	30.72 ± 0.29	26.40 ± 0.20*	22.23 ± 0.37$^{*,\#}$	21.59 ± 0.67$^{*,\#}$
Energy intake (kcal/rat/day)	92.85 ± 0.69	154.90 ± 2.81*	124.30 ± 3.16$^{*,\#}$	120.90 ± 2.57$^{*,\#}$
Fasting blood glucose (mg/dL)	78.83 ± 5.65	103.00 ± 3.14*	85.00 ± 2.35$^{\#}$	82.83 ± 2.82$^{\#}$
AUC of blood glucose (min.mg/dL)	14550 ± 176	19337 ± 758*	14847 ± 288$^{\#}$	16242 ± 480$^{\#}$
Serum insulin (ng/mL)	2.43 ± 0.69	9.12 ± 0.58*	3.70 ± 0.45$^{\#}$	3.56 ± 0.52$^{\#}$
HOMA-IR	11.27 ± 3.16	57.85 ± 4.99*	19.35 ± 2.63$^{\#}$	18.02 ± 2.62$^{\#}$
Serum adiponectin (µg/mL)	20.83 ± 0.95	22.68 ± 0.72	31.77 ± 1.42$^{*,\#}$	27.86 ± 1.38$^{*,\#}$
Serum leptin (ng/mL)	16.19 ± 0.32	41.12 ± 0.81*	26.01 ± 1.24$^{*,\#}$	17.46 ± 1.64$^{\#}$
Serum free fatty acid (mg/dL)	112.54 ± 5.21	175.31 ± 11.52*	80.77 ± 8.33$^{\#}$	103.92 ± 10.77$^{\#}$
Serum triglyceride (mg/dL)	71.58 ± 3.82	105.88 ± 13.42*	47.13 ± 5.03$^{\#}$	61.08 ± 3.44$^{\#}$
Serum LDL-C (mg/dL)	14.36 ± 0.34	17.38 ± 0.32*	14.25 ± 0.58$^{\#}$	14.67 ± 0.45$^{\#}$
Serum HDL-C (mg/dL)	32.34 ± 0.91	21.50 ± 0.61*	27.22 ± 1.43$^{*,\#}$	32.06 ± 1.17$^{\#}$

Results are expressed as means ± SEM (n = 6). *p < 0.05 versus ND group, #p < 0.05 versus HFFD group; ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin; HOMA-IR, Homeostasis model assessment of the insulin resistance index.

Effects of 3-CQA on metabolic parameters

As presented in **Table 2**, rats in the HFFD control group had a significant increase in body weight compared to the normal diet (ND) control group (p < 0.05). The gain in body weight of rats in groups treated with 3-CQA or metformin was significantly lower than in the HFFD group. The food intake of the HFFD group was significantly lower, whereas the energy intake was significantly higher than in the ND group (p < 0.05) (**Table 2**). Interestingly, HFFD rats treated with 3-CQA or metformin exhibited a significant decrease in both of the daily food and energy intakes compared to the HFFD control group (p < 0.05). These results demonstrated that 3-CQA treatment could prevent obesity in rats fed the HFFD.

FBG, AUC of blood glucose, serum insulin, and HOMA-IR score were all significantly high in rats in the HFFD control group compared to the ND group (p < 0.05), confirming an insulin-resistant condition in the former. 3-CQA or metformin treatments significantly reduced all of these parameters (p < 0.05) (**Table 2**).

Additionally, serum leptin levels were increased in the HFFD group, whereas serum adiponectin levels were not changed in comparison to the ND group. 3-CQA and metformin treatments significantly reduced serum leptin levels and increased adiponectin levels compared to the HFFD group (p < 0.05) (**Table 2**).

Effects of 3-CQA on lipid profiles, **Table 2** shows that the HFFD control animals had significantly higher levels of serum FFA, TG, and LDL-C, and lower level of HDL-C than those in the ND group (p < 0.05). However, the levels of FFA, TG, and LDL-C were decreased, and HDL-C was increased in the

groups treated with 3-CQA or metformin compared to the HFFD control group ($p < 0.05$). Thus, treatment with 3-CQA resulted in improvement of lipid profiles.

Effects of 3-CQA on adipose tissue weight and adipocyte size

As shown in **Figures 1(A) - 1(C)**, the visceral adipose tissue weight and the adipocyte size in the HFFD control group were significantly greater than those in the ND group ($p < 0.05$). In HFFD rats treated with 3-CQA or metformin, visceral adipose tissue weight and the adipocyte size were significantly lower than in the HFFD control group ($p < 0.05$). These results indicated that 3-CQA treatment suppressed lipid deposition in adipocytes.

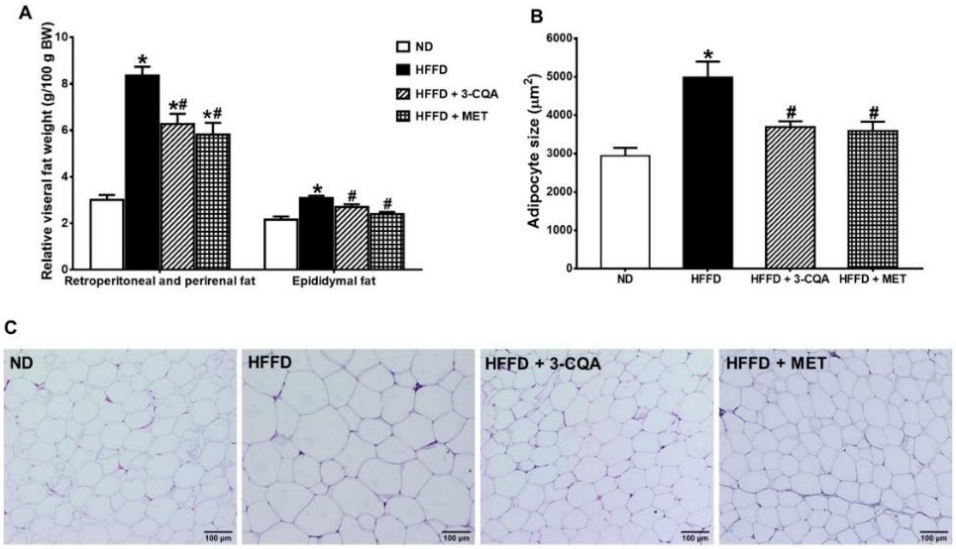

Figure 1 Effects of 3-CQA on (A) relative weight of visceral fat tissue: retroperitoneal, perirenal and epididymal fat tissues, (B) average size of adipocytes, and (C) histological images of adipocytes of epididymal fat tissue (H&E staining, 200 × magnification). Results are expressed as means ± SEM ($n = 6$). *$p < 0.05$ versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on oxidative stress markers

As shown in **Figures 2(A) - 2(C)**, serum MDA levels were significantly increased ($p < 0.05$) and the activities of SOD and CAT were significantly decreased in the HFFD control group ($p < 0.05$) relative to the ND group. The MDA levels in the HFFD rats treated with 3-CQA or metformin were significantly lower, and the activities of SOD and CAT were significantly higher than in the HFFD control group ($p < 0.05$). These indicated that 3-CQA attenuated oxidative stress in rats induced by HFFD.

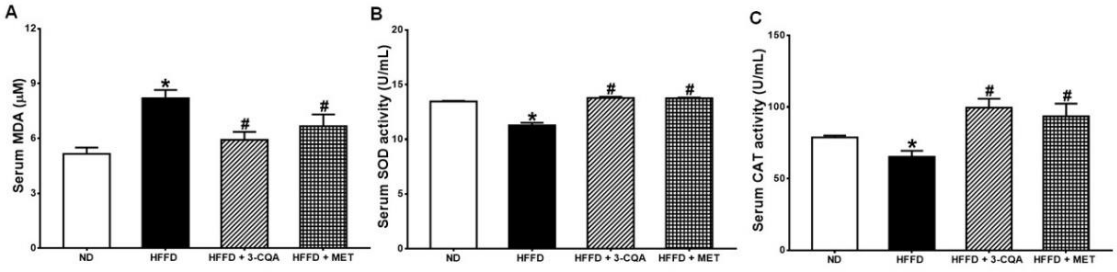

Figure 2 Effects of 3-CQA on (A) serum MDA level, (B) serum SOD, and (C) serum CAT activities. Results are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on expression of pro-inflammatory mediator mRNA in epididymal adipose tissue

In the HFFD control group, the mRNA expression levels of *MCP-1*, *TNF-α*, and *IL-6* were significantly upregulated compared to the ND group ($p < 0.05$). However, they were significantly downregulated in 3-CQA- or metformin-treated groups (**Figures 3(A) - 3(C)**) ($p < 0.05$), suggesting that 3-CQA treatment could inhibit the inflammation of adipose tissue in HFFD-fed rats.

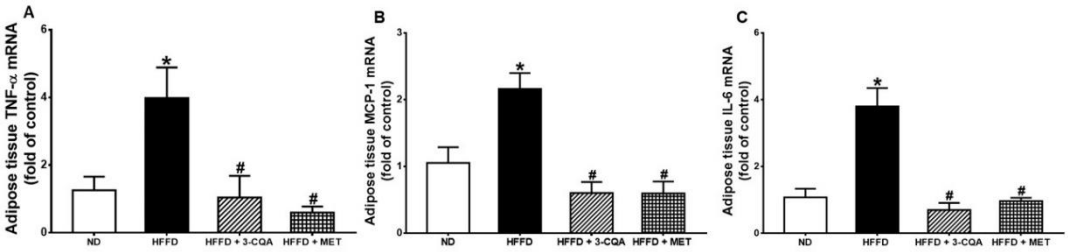

Figure 3 Effects of 3-CQA on mRNA expression of pro-inflammatory mediator genes; (A) *TNF-α*, (B) *MCP-1*, and (C) *IL-6* in epididymal adipose tissue. Results are expressed as means ± SEM ($n = 6$). *$p <$ 0.05 versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on liver weight and fat accumulation in liver

The relative liver weight, hepatic TG content, and degree of lipid droplet accumulation were significantly greater in the HFFD group compared to the ND group ($p < 0.05$) (**Figures 4(A) - (C)**). 3-CQA or metformin-treated groups had a significantly lower accumulation of TG in the liver (**Figure 4(B)**) and histo-microscopic examination showed a decrease of lipid droplets in the liver as well (**Figure 4(C)**). However, liver weights in the 3-CQA and MET groups did not differ significantly from those in the HFFD group ($p < 0.05$, **Figure 4(A)**). These results showed that 3-CQA treatment could suppress lipid accumulation in liver tissues.

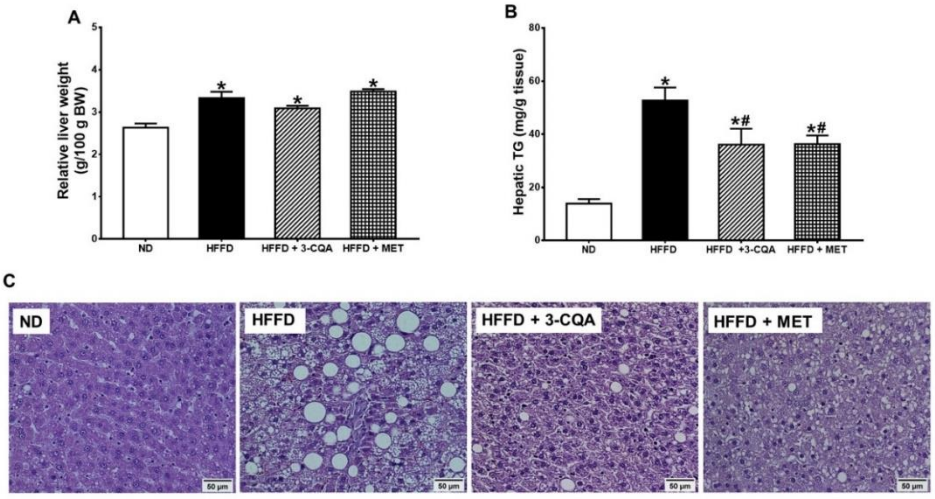

Figure 4 Effects of 3-CQA on (A) liver weight, (B) hepatic triglyceride content, and (C) representative histological images of liver (H&E staining, 200 × magnification). Results are expressed as means ± SEM ($n = 6$). *$p <$ 0.05 versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on the expression of glucose metabolism-related genes

Rats in the HFFD control group had significantly greater expression levels of hepatic *PEPCK* and *G6Pase* mRNA, but lower expression levels of muscle *GLUT4* mRNA compared to the ND rats ($p < 0.05$). Treatment of HFFD rats with 3-CQA or metformin significantly reversed mRNA expression levels of these

glucose metabolism-related genes compared to the HFFD control group ($p < 0.05$) (**Figures 5(A) - 5(C)**). These results suggested that 3-CQA treatment could improve an impaired glucose metabolism by suppressing hepatic gluconeogenic genes in the liver and enhancing expression of glucose transporter gene in the muscle tissues.

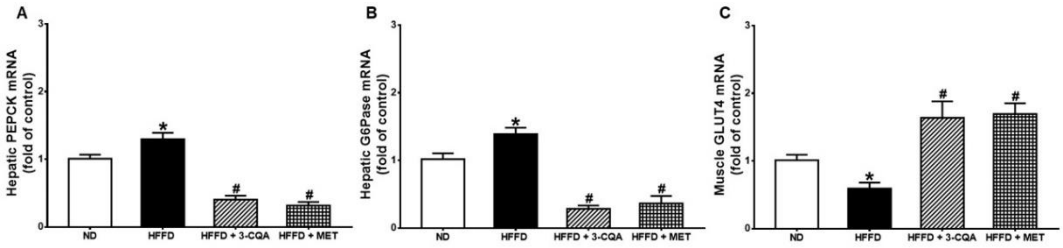

Figure 5 Effects of 3-CQA on the expression of glucose metabolism-related genes; (A) *PEPCK* and (B) *G6Pase*, and (C) muscle *GLUT4* mRNA. Results are expressed as means ± SEM ($n = 6$). *$p < 0.05$ versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on the expression of lipid metabolism-related genes

In the HFFD control group, the mRNA expression levels in the liver of lipid synthesis-regulating genes: *SREBP1c, FAS*, and *GPAT* were significantly increased (**Figure 6(A)**), and fatty-acid oxidation-regulating genes: *PPARα, CPT1*, and *ACOX1* were significantly decreased compared with the expression levels in the ND group ($p < 0.05$) (**Figure 6(B)**). The increases in expression levels of *SREBP1c*, *FAS*, and *GPAT* mRNA induced by the HFFD were significantly decreased after 3-CQA or metformin treatments ($p < 0.05$). However, non-significant changes in expression levels of *PPARα*, *CPT1*, and *ACOX1* mRNA (relative to the HFFD group) were observed after 3-CQA or MET treatments. These results indicated that 3-CQA ameliorated intrahepatic fat accumulation which may be associated with the decrease in expression levels of lipid synthesis-related genes, but not be involved in fatty-acid oxidation processes.

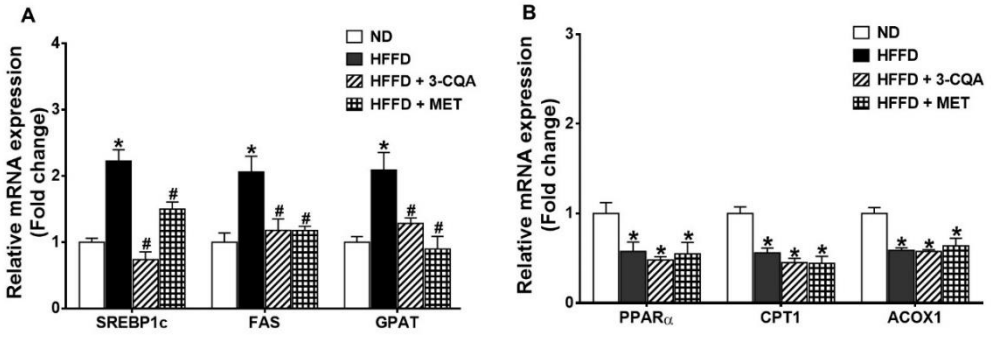

Figure 6 Effects of 3-CQA on the expression of hepatic lipid metabolism-related genes; (A) *SREBP1c*, *FAS*, and *GPAT*; and (B) *PPARα*, *CPT1*, and *ACOX1* mRNA. Results are expressed as means ± SEM ($n = 6$). *$p < 0.05$ versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Effects of 3-CQA on protein expression of p-AKT, p-AMPK, and GLUT4 in the skeletal muscle

The results (**Figures 7(A) - (C)**) showed that the ratio of p-AKT/AKT and p-AMPK/AMPK were lowered in the HFFD control group compared with the ND group ($p < 0.05$). Interestingly, treatment for 6 weeks with 3-CQA or metformin significantly enhanced the ratio of p-AKT/AKT and p-AMPK/AMPK in the skeletal muscle ($p < 0.05$).

As presented in **Figure 7(D)**, GLUT4 was detected in cross-sections of skeletal muscle by immuno-histochemical staining shown in brown color. A reduction in GLUT4 immunostaining was observed in muscle sections of HFFD group. Treatment with 3-CQA or metformin could increase the expression of GLUT4 protein in the muscle compared with the HFFD control group. The GLUT4 staining was quantified

using ImageJ Fiji software. Interestingly, 3-CQA or MET treatment significantly increased the intensity of staining of GLUT4 compared with the HFFD control group ($p < 0.05$) (**Figure 7(E)**).

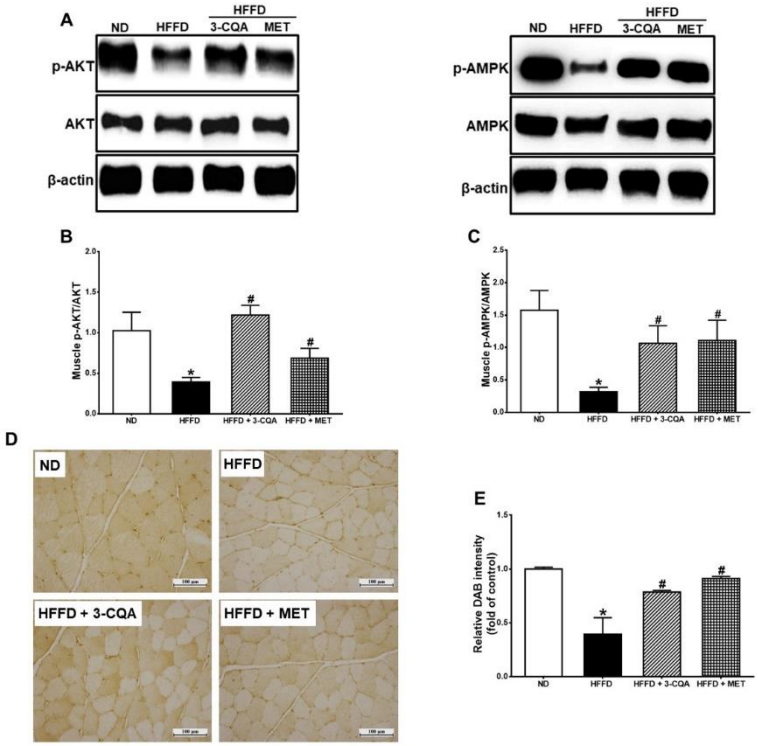

Figure 7 Effects of 3-CQA on (A) protein expression of p-AKT, AKT, p-AMPK, and AMPK, (B) ratio of p-AKT and AKT and (C) ratio of p-AMPK and AMPK in skeletal muscle, (D) representative micrographs of immuno-histochemical staining of GLUT4, and (E) quantification of staining intensity of GLUT4 by ImageJ Fiji in skeletal muscle. Results are expressed as means ± SEM ($n = 3 - 5$). *$p < 0.05$ versus ND group, #$p < 0.05$ versus HFFD group. ND, Normal diet control group; HFFD, High fat-high fructose diet control group; 3-CQA, 3-caffeoylquinic acid; MET, Metformin.

Discussion

The CQAs were commonly known as important plant constituents in fruits and vegetables [14]. Many previous studies revealed anti-obesity, hypoglycemic and hypolipidemic properties of 5-CQA in animal experiments [26]. The present study demonstrates the effects of 3-CQA on impaired glucose and lipid metabolism in the insulin-resistant condition in rats fed the HFFD. 3-CQA treatment improved insulin sensitivity and ameliorated body weight gain, dyslipidemia, hepatic steatosis, oxidative stress, and inflammation of adipose tissue in the HFFD-fed rats. These findings were consistent with *in vitro* experiments showing hypoglycemic and hypolipidemic activities of 3-CQA [16,17]. At molecular level, 3-CQA treatment suppressed the expression of hepatic gluconeogenic genes and lipid synthesis-related genes, increased expression of muscle *GLUT4* genes and protein, and upregulated the phosphorylation levels of AKT and AMPK in skeletal muscle.

Administration of 3-CQA lowered the body-weight gain together with the decreases in food intake and energy intake, this implies that 3-CQA treatment suppresses HFFD-induced obesity, at least in part, by reducing food or energy intake. Leptin, an adipocyte-derived adipokine, plays important roles in the regulation of food intake and energy expenditure. Leptin resistance leads to high circulating leptin levels and failure to promote anorectic and weight-reducing actions in the obese state [27]. Our experiment demonstrated the anti-obesity action of 3-CQA may be associated with the restoration of leptin sensitivity. The leptin resistant phenomenon may be induced by several underlying mechanisms, such as serum leptin-interacting proteins (SLIPs) and soluble leptin receptor (SLR) may bind circulating adipose-secreted leptin resulting in inhibiting leptin action, and also a down regulation of leptin receptor [28]. From our experiments, it is still quite hard to indicate the precise mechanism of 3-CQA in the restoration of leptin sensitivity.

Insulin resistance results in decreased blood-glucose disposal, increased lipolysis, and hepatic glucose production accompanied by hyperinsulinemia [29]. Our results showed 3-CQA treatment could alleviate these alterations: Decreased blood glucose, improved glucose tolerance and insulin sensitivity, and decreased serum insulin levels. These results suggest that 3-CQA can increase insulin sensitivity. In addition, levels of adiponectin increased with 3-CQA treatment. It enhances peripheral effects of insulin in target tissues and anti-inflammatory response. The important effects of adiponectin are suppressing hepatic glucose production by reducing the rate-limiting enzymes of gluconeogenesis, such as G6Pase and PEPCK, and enhancing glucose uptake by adipocytes and skeletal muscle cells [30]. Under insulin-resistant condition, a reduction in the glucose uptake (either through decreased expression of *GLUT4* gene or decreased translocation of GLUT4) into the muscle and adipose tissues, and an increase in hepatic glucose production by enhanced expression of PEPCK and G6Pase lead to the increase in the blood glucose levels [25,29]. After 6 weeks of 3-CQA treatment, downregulation of *PEPCK* and *G6Pas*e genes in the liver, and upregulation of *GLUT4* genes in skeletal muscle were found together with an increase in serum levels of adiponectin. Thus, one of the mechanisms by which 3-CQA improves insulin sensitivity and glucose metabolism in target tissues may be associated with increased adiponectin secretion.

Oxidative stress, an imbalance between the oxidant production and antioxidant defenses, is the key contributor to insulin resistance [31]. Previous findings showed an *in vitro* antioxidant activity of 3-CQA [32]. Here, we demonstrated that 3-CQA treatment can decrease serum MDA levels, and increase the activity of serum antioxidants (SOD and CAT) in HFFD-fed rats, suggesting that 3-CQA treatment enhances the antioxidant defense mechanism and thereby resulting in improved insulin sensitivity.

White adipose tissue (WAT) is a major storage site for excess energy in the form of TG in adipocytes and release of FFA for use by other organs as a source of energy. During the development of obesity, WAT expands through an increase in fat-cell size (hypertrophy) and/or fat-cell number (hyperplasia). Subsequently, hypertrophic adipocytes show altered secretion of adipokines and enhanced basal lipolysis, resulting in increased release of FFA. Many pro-inflammatory adipokines such as MCP-1, TNF-α, and, IL-6, etc. are secreted from expanded adipose tissue, which leads to inflammation of adipose tissue and decreased insulin sensitivity [9]. Interestingly, 3-CQA treatment decreased the weight of WAT and size of adipocytes, serum level of FFA and especially gene expression levels of *MCP-1*, *TNF-α*, and *IL-6* in WAT. These findings imply that 3-CQA prevented HFFD-induced adipocyte hypertrophy and adipose tissue inflammation, eventually leading to an increase in insulin sensitivity.

Increased lipolysis from adipose tissue results in increased release of FFA into circulation and an influx to the liver. FFAs are then re-esterified as TG in the liver and released as VLDL-C, leading to hypertriglyceridemia, formation of small dense low-density lipoprotein-cholesterol and decreased HDL-C levels, which are characteristics of dyslipidemia induced by insulin resistance [33]. As well, the consumption of a high-fat diet causes an increased delivery of dietary fatty acids to the liver that exceeds the liver disposal capacity, leading to an accumulation of hepatic TG or NAFLD [34]. Overconsumption of fructose also induces NAFLD by its ability to upregulate *de novo* lipogenesis (DNL). Fructose-induced DNL generates fatty acids that can then be incorporated into hepatic TG or other lipid species, and fructose also independently activates SREBP1c, which in turn activates DNL [35]. In this study, we found that 3-CQA treatment improved dyslipidemia by lowering serum FFA, TG and LDL-C levels, and increasing the level of HDL-C. In addition, treatment with 3-CQA effectively reduced these hepatic TG levels and lowered the numbers of lipid droplets. These findings indicated that 3-CQA treatment ameliorates dyslipidemia and NAFLD in HFFD-fed rats.

SREBP1c is a transcription factor in hepatic lipogenesis by stimulating transcription of lipogenic enzymes, such as fatty-acid synthase (FAS) and glycerol-3-phosphate acyltransferase (GPAT), the key enzymes in fatty acid and TG synthesis [36]. Hyperinsulinemia and fructose intake stimulate hepatic SREBP1c transcription resulting in increased hepatic lipid synthesis leading to development of fatty liver [35,36]. PPARα plays an important role in the regulation of hepatic lipid metabolism by transcriptional activation of key rate-limiting enzymes of fatty-acid oxidation, such as carnitine palmitoyl transferase 1 (CPT1) and acyl-CoA oxidase 1 (ACOX1). CPT1 is the enzyme involved in mitochondrial fatty acid β-oxidation, and ACOX1 catalyzes the first step in hepatic peroxisomal β-oxidation [38]. There has been a report showed that hepatic expression of PPARα is decreased in NAFLD, but it increases when the histological picture of NAFLD is improved [39]. Previous study in cultured hepatic cells showed that 3-CQA could inhibit lipid synthesis-related genes; *SREBP1c* and *FAS*, while it increased fatty-acid oxidation-related genes; *PPARα*, *CPT1* and *ACOX1* in HepG2 cells with lipid accumulation induced by oleic acid [17]. However, in the present study, we found that 3-CQA could lower only the expression of lipid synthesis-related genes, but not of those related to fatty-acid oxidation. This may be because of the difference in experimental circumstances; *in vitro versus in vivo*. Thus, we conclude that 3-CQA may

decrease lipid genesis, and it then ameliorates dyslipidemia and NAFLD by downregulation of genes involved in hepatic *de novo* lipogenesis.

There are 2 distinct pathways involved in stimulated glucose uptake by skeletal muscle cells. One is directly activated by insulin through phosphatidylinositol 3-kinase/AKT signaling (PI3K/AKT) pathway and the other is through stimulation by AMP-activated protein kinase (AMPK), a principal regulator of intracellular energy sensors [40]. In response to insulin, phosphorylated AKT causes GLUT4 translocation to the plasma membrane and uptakes glucose into skeletal muscle [41]. Furthermore, activated AMPK triggers GLUT4 trafficking to the plasma membrane and increases glucose uptake in skeletal muscles [42]. Thus, upregulation of AKT or AMPK phosphorylation may lead to increase in GLUT4 expression in skeletal muscle [43]. Previous studies demonstrated that a high-calorie diet causes a decrease in AKT and/or AMPK phosphorylation and expression of *GLUT4* mRNA and protein levels, resulting in impaired insulin sensitivity at both whole-body and skeletal muscle levels [43,44]. Interestingly, 3-CQA restored the downregulated AKT and AMPK phosphorylation along with increased the levels of both *GLUT4* mRNA and protein expression in skeletal muscle in HFFD-fed rats. These findings indicated that 3-CQA may improve insulin response through enhancing glucose uptake by activating AKT and AMPK pathways.

Metformin, an insulin sensitizer commonly used for the treatment of type 2 diabetes, induces glucose-lowering effect through the activation of AMPK. AMPK activation by metformin can suppress hepatic glucose production and promote glucose uptake in skeletal muscles, and also can suppress hepatic lipogenesis [45]. As well, our study showed that metformin attenuated insulin resistance through the activation of AKT pathway, which was consistent with a previous report [46]. Interestingly, 3-CQA exerted the same metabolic improvements in HFFD-fed rats as metformin.

Conclusions

In conclusion, 3-CQA could improve glucose and lipid homeostasis in HFFD-fed rats by decreasing insulin resistance which may be due to its activities in ameliorating obesity, oxidative stress, and adipose tissue inflammation. The molecular mechanisms of these beneficial effects of 3-CQA may be mediated via (a) the suppression of expression of hepatic gluconeogenic (*PEPCK* and *G6Pase*) and hepatic lipogenic (*SREBP1c*, *FAS*, and *GPAT*) genes, and (b) increased expression of muscle *GLUT4* gene and GLUT4 protein. Moreover, the effect of 3-CQA on insulin sensitivity may be associated with activation of AKT and AMPK pathways.

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