Dietary chlorogenic acid ameliorates oxidative stress and improves endothelial function in diabetic mice via Nrf2 activation

Dan Wang¹,²,* Jixin Hou¹,²,* Jindong Wan¹,², Yi Yang¹,², Sen Liu¹,², Xiaoqing Li³, Wenzhang Li¹,², Xiaozhen Dai³, Peng Zhou¹,², Weihua Liu⁵ and Peijian Wang¹,²

Abstract

Objectives: Chlorogenic acid (CGA) is an antioxidant dietary factor. We investigated the effects of CGA on endothelial cell dysfunction in diabetic mice and the mechanistic role of nuclear factor erythroid-related factor 2 (Nrf2) in the antioxidant effect of CGA.

Methods: Diabetic (db/db) mice were fed normal chow or chow containing 0.02% CGA for 12 weeks. Human umbilical vein endothelial cells (HUVECs) and mouse aortas were treated with normal or high glucose.

Results: CGA treatment induced upregulation of Nrf2 in HUVECs in a dose-dependent manner. CGA pretreatment prevented reactive oxygen species generation and preserved nitric oxide bioavailability in HUVECs and aortas from wild-type but not Nrf2⁻/⁻ mice. CGA improved endothelium-dependent relaxation in high glucose-treated aortas from wild-type and db/db mice, but not Nrf2⁻/⁻ mice. Dietary CGA improved endothelium-dependent relaxation in db/db mice.

Conclusions: CGA ameliorates endothelial dysfunction in diabetic mice through activation of the Nrf2 anti-oxidative pathway.

¹Department of Cardiology, The First Affiliated Hospital, Chengdu Medical College, Chengdu, Sichuan, China
²Key Laboratory of Aging and Vascular Homeostasis, Sichuan Higher Education Institute, The First Affiliated Hospital, Chengdu Medical College, Chengdu, Sichuan, China
³School of Pharmacy, Chengdu Medical College, Chengdu, Sichuan, China
⁴School of Biological Sciences and Technology, Chengdu Medical College, Chengdu, Sichuan, China
⁵Department of Scientific Research, The First Affiliated Hospital, Chengdu Medical College, Chengdu, Sichuan, China

*These authors contributed equally to this work.

Corresponding author:
Peijian Wang, Department of Cardiology, The First Affiliated Hospital, Chengdu Medical College, 278 Baoguang Ave, Chengdu, Sichuan 610500, China.
Email: wpjmed@aliyun.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
Introduction

Diabetes is a major risk factor for cardiovascular disease. Between 70% and 80% of diabetic patients will eventually die from cardiovascular complications, imposing a heavy financial burden on society. Glucose toxicity, low-grade inflammation, and oxidative stress induce insulin resistance, damage pancreatic β cells, and cause endothelial dysfunction, ultimately resulting in micro- and macro-vascular complications. Unfortunately, there is no specific treatment for diabetic endothelial dysfunction.

Oxidative stress is a condition in which reactive oxygen species (ROS) production exceeds that of antioxidants. Oxidative stress is a critical contributor to diabetic vascular injury. Increased intracellular ROS levels can induce apoptosis of endothelial cells. As a redox-sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) exerts antioxidant actions by inducing expression of antioxidant enzymes. Under physiologic conditions, Nrf2 is found in the cytoplasm at low concentration. Under stressful conditions, Nrf2 translocates into the nucleus and acts as a transcription factor to maintain redox homeostasis. Recent studies have revealed that Nrf2 plays an important role in diabetic vascular injury and that targeting Nrf2 may be a promising strategy for treatment of diabetes-related cardiovascular complications.

Chlorogenic acid (CGA) is a polyphenolic compound that is abundant in coffee, strawberries, and sunflowers. CGA possesses many biological properties, including antioxidant and anti-carcinogenic activities. Recently, the roles of CGA in glucose and lipid metabolic disorders have also been investigated. Dietary administration of CGA attenuated isoproterenol-induced oxidative stress in the rat myocardium. CGA directly induces endothelium-dependent vasodilation in isolated rat vessels. Although CGA has consistently been shown to inhibit oxidative stress and exert cardioprotective effects, its role in diabetes-induced endothelial dysfunction remains unknown. The present study aimed to test the hypothesis that CGA attenuates diabetes- and high-glucose-induced endothelial dysfunction via Nrf2-mediated antioxidant effects.

Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chengdu Medical College (2018013). Male Nrf2 knockout mice (Nrf2−/−, 12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Male leprdb diabetic (db/db) mice and age-matched wild-type control mice (C57BL/KsJ) were purchased from the Model Animal Research Center (Nanjing, Jiangsu, China). Mice were housed under a 12-hour/12-hour light/dark cycle with free access to food and water.
water. Mice were fed normal chow (control group) or normal chow containing 0.02% CGA (Sigma-Aldrich, St. Louis, MO, USA) (CGA group) for 12 weeks.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Schenkel Research Laboratories (San Diego, CA, USA). HUVECs were grown in serum- and supplement-free Dulbecco’s Modified Eagle’s Medium (DMEM, HyClone, Logan, UT, USA) containing normal glucose (NG, 5 mM) for 24 hours. Then, cells were either maintained in serum-free NG DMEM (containing 25 mM mannitol as an osmotic control) or switched to serum-free high glucose (HG, 30 mM) DMEM with or without CGA (10\,\mu M, diluted in dimethyl sulfoxide). The nuclear fractions were prepared using NE-PER® Nuclear Extraction Reagents (Thermo Scientific, Waltham, MA, USA).

Delivery of small interfering RNA (siRNA)

HUVECs were cultured in medium containing 20 \,\mu g of Nrf2 siRNA or scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 6 hours per day on 2 consecutive days according to the manufacturer’s instructions.

Measurement of vascular tone

Aortic rings from WT and Nrf2\(^{-/-}\) mice were dissected in sterile phosphate-buffered saline and incubated in DMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 IU/mL penicillin, and 100\,\mu g/mL streptomycin. After a 24-hour incubation period, a wire myograph was used to assess vascular reactivity of the aortic rings.\(^{16}\) An optimized baseline tension was applied to the arterial rings, and arterial segments were balanced for 1 hour. The rings were pre-contracted with phenylephrine (1\,\mu M), then endothelium-dependent relaxation in response to acetylcholine (ACh, 10\(^{-9}\) M – 10\(^{-5}\) M) and endothelium-independent relaxation in response to nitroglycerine (NTG, 10\(^{-9}\) M – 10\(^{-5}\) M) were recorded.

Determination of ROS

Dihydroethidium (DHE, Sigma-Aldrich) was used to detect superoxide as described previously.\(^{17}\) In situ formation of ROS was measured by DHE staining. DHE staining was visualized using a fluorescence microscope (TE2000, Nikon, Toyoko, Japan). Images were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Evaluation of nitric oxide (NO) levels

HUVECs and aortic segments were washed three times in Krebs solution and stained with diaminofluorescein-2 diacetate (DAF-2DA, a NO-sensitive fluorescent dye; Sigma-Aldrich). After incubation for 45 minutes at 37°C, DAF-2 DA fluorescence intensity was analyzed using NIS-Elements 3.0 software (Nikon).\(^{18}\)

Measurement of protein expression

Membranes were incubated with primary antibodies against Nrf2 (Abcam, Cambridge, MA, USA), nitrotyrosine (Santa Cruz Biotechnology), quinone oxidoreductase 1 (NQO1, Santa Cruz), heme oxygenase-1 (HO-1, Santa Cruz), P22phox (Santa Cruz), P47phox (Santa Cruz), P67phox (Santa Cruz), glutathione peroxidase 1 (GPx-1, Santa Cruz), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz), and Histone H1 (Abcam). Blotted membranes were washed and incubated with secondary antibodies (ZSGB-Bio, Beijing, China) for 2 hours at room temperature. Protein levels were normalized to levels of GAPDH or Histone H1.
Blots were visualized using a Gel Doc 2000 Imager (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data were presented as means ± standard errors of the means (SEMs). Maximum response \( \left( E_{\text{max}} \right) \) was calculated based on concentration-response curves using GraphPad Prism software version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences in the means of three or more groups were assessed using one-way analysis of variance with post-hoc Tukey’s honestly significant difference test. Differences between the means of two groups were assessed using the Student’s \( t \)-test. Values of \( P < 0.05 \) were considered statistically significant.

Results

CGA upregulated Nrf2 and downregulated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in HUVECs

Previous studies reported a Nrf2-mediated cytoprotective effect of CGA, but the role of CGA in endothelial cell function remains unknown. We first investigated the effect of CGA on Nrf2 expression in HUVECs. Treatment with CGA increased nuclear Nrf2 expression in HUVECs in a dose-dependent manner. The effects of 10 \( \mu \)M and 100 \( \mu \)M CGA doses were similar (Figure 1a). As shown by western blotting, CGA (10 \( \mu \)M) also upregulated expression of the Nrf2 downstream targets HO-1, NQO1, and GPx1 (Figure 1b–1e). CGA (10 \( \mu \)M) pretreatment decreased the expression of the NADPH oxidase subunits P22\( \text{phox} \), P47\( \text{phox} \), and P67\( \text{phox} \), and reduced the generation of nitrotyrosine, a marker of ONOO\(^- \)-mediated nitration that plays a critical role in vascular injury under hyperglycemic conditions. CGA also upregulated Nrf2 expression in HG-treated HUVECs (Figure 2a). Furthermore, CGA attenuated HG-induced upregulation of P22\( \text{phox} \), P47\( \text{phox} \), and P67\( \text{phox} \), as well as HG-induced generation of nitrotyrosine (Figure 2b–2f). The beneficial effects of CGA were abrogated by Nrf2 silencing using siRNAs.

CGA decreased superoxide levels and preserved NO levels in HUVECs

NO plays a pivotal role in protection of endothelial cells. Loss of endothelium-derived NO is a hallmark of endothelial dysfunction and is implicated in the pathogenesis of cardiovascular disease. We next asked whether the effects of CGA on Nrf2 and its downstream targets impacted NO levels in HUVECs cultured under HG conditions. CGA pretreatment prevented superoxide anion generation (Figure 3a and 3c) and preserved NO levels in HG-treated HUVECs (Figure 3b and 3d), as shown by DHE and DAF-2DA staining, respectively. The beneficial effects of CGA were abrogated by Nrf2 silencing using siRNAs (Figure 3). These results suggested that CGA prevents HG-induced ROS generation and preserves NO bioavailability in HUVECs through an Nrf2-dependent pathway.

CGA preserved the endothelial function of mouse aortas via Nrf2

Aortic rings were isolated from wild-type and Nrf2\(^-/- \) mouse aortas and cultured in NG, HG, or HG medium containing CGA. CGA (10 \( \mu \)M) reversed HG-induced increases in ROS production and decreases in NO levels in aortas from wild-type but not Nrf2\(^-/- \) mice (Figure 4). Moreover, we found that CGA ameliorated impairment of ACh-induced endothelium-dependent relaxation under HG conditions in cultured aortic rings from wild-type (Figure 5a) but
not Nrf2−/− mice (Figure 5b). We observed no effect of CGA on NTG-induced endothelium-independent relaxation in either wild-type or Nrf2−/− mice (Figure 5c and 5d). These results suggested that CGA preserves endothelial function under HG conditions in an Nrf2-dependent manner.

**Chronic CGA administration improved endothelium function in diabetic mice via Nrf2**

Diabetic (db/db) mice and age-matched wild-type mice were used to explore the effects of CGA on endothelial dysfunction...
After 12 weeks of CGA (0.02%) feeding, db/db mice had decreased body weights (Figure 6a), fasting blood glucose (Figure 6b), and hemoglobin A1c levels (Figure 6c) compared with control mice. Dietary CGA attenuated ROS production and increased NO levels in the endothelium of db/db mice (Figure 6d–6g). A wire myograph was used to assess relaxation of mouse aortas. Twelve weeks of CGA feeding improved ACh-induced aortic relaxation in db/db mice (Figure 7a), an effect that was blocked by the endothelial NO synthase inhibitor L-NAME (Figure 7b).

**Figure 2.** Effects of chlorogenic acid (CGA) on P22phox, P47phox, P67phox and nitrotyrosine levels in human umbilical vein endothelial cells (HUVECs) cultured under high glucose (HG) conditions. Histone H1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were used as a reference. (a) Exposure to HG (30 mM) increased the expression of Nrf2, and Nrf2 expression was further elevated by CGA treatment. (b–f) HG (30 mM) increased P22phox, P47phox, P67phox and nitrotyrosine levels, while CGA pretreatment decreased P22phox, P47phox, P67phox and nitrotyrosine levels under HG conditions. These effects were blocked by siRNA silencing of Nrf2. **P < 0.01 compared with the normal glucose (NG, 5 mM) group; ###P < 0.01 compared with the HG group; △△P < 0.01 compared with the HG+CGA group (n = 6).**
Dietary CGA did not affect NTG-induced relaxation in aortic rings from db/db mice (Figure 7c). Western blotting revealed that CGA feeding attenuated downregulation of Nrf2, upregulation of P22phox, P47phox, and P67phox, and generation of nitrotyrosine in db/db mice (Figure 8a–8f).

**Discussion**

The present study showed that under HG conditions, CGA upregulated Nrf2 and its downstream targets, downregulated P22phox, P47phox, and P67phox, prevented ROS and nitrotyrosine production, and
Figure 4. Effects of chlorogenic acid (CGA) on reactive oxygen species (ROS) production and nitric oxide (NO) levels in mouse aortas treated with high glucose (HG) ex vivo. (a, b, e) ROS production was measured by dihydroethidium (DHE) staining in the en face endothelium of aortas from Nrf2 knockout (Nrf2−/−) and matched wild type (WT) mice after treatment with normal glucose (NG), HG or HG+CGA (10 μM). (c, d, f) NO production in the en face endothelium of aortas was measured by diaminofluorescein-2 diacetate (DAF-2DA) staining. Red: DHE fluorescence. Green: DAF-2DA fluorescence. **p < 0.01 compared with the NG group; ###p < 0.01 compared with the HG group. Data are presented as means ± SEMs (n = 6). Scale bar, 20 μm.
preserved NO levels in HUVECs. Similar effects were observed in cultured aortic rings from wild-type mice but not Nrf2−/− mice. Furthermore, an in vivo study demonstrated that dietary CGA increased the expression of antioxidant enzymes (HO-1, GPx-1, and NQO1) and improved endothelial function. These findings suggest that CGA ameliorates endothelial dysfunction in diabetic mice.

Oxidative stress is closely associated with the pathogenesis of diabetes-related cardiovascular complications. CGA, one of the most abundant polyphenol compounds in the human diet, belongs to a group of phenolic secondary metabolites produced by certain plant species and is a major component of coffee. CGA has been shown to attenuate oxidative stress both in models of oxidized low-density lipoprotein-induced endothelial dysfunction and streptozotocin-induced diabetes in rats. Additionally, the protective effects of CGA against oxidative stress-induced cell damage were associated with the Nrf2 signaling pathway. However, whether CGA protects against...
hyperglycemia-induced endothelial cell injury was unclear, and its effects on endothelial dysfunction in diabetes were unknown. In the present study, we showed that CGA increased nuclear Nrf2 expression in a dose-dependent manner.

Absorption and metabolism of CGA have been studied in healthy volunteers and human tissues. Because there is no evidence that these dietary conjugates are absorbed in vivo and true bioavailability data are incomplete, previously determined concentrations of CGA may be inaccurate. Despite these caveats, high levels of CGA are potentially achievable in human tissues.
blood. Our study is the first to assess the effects of CGA on Nrf2 in endothelial cells.

Nrf2, a transcription factor with a high sensitivity to oxidative stress, binds to antioxidant responsive elements in the nucleus and promotes transcription of a wide variety of antioxidant genes. HO-1, NQO1, and GPx1 are the major downstream antioxidant effectors of Nrf2. Upregulation and activation of Nrf2 increases the expression of HO-1, NQO1, and GPx1 through positive transcriptional regulation. In the present study, we found that CGA upregulated the expression of HO-1, NQO1, and GPx1 in addition to Nrf2. These results suggest that CGA enhances expression of anti-oxidative genes including HO-1 via upregulation of Nrf2. This finding is consistent with a previous study demonstrating that CGA activated the Nrf2/HO-1 anti-oxidative pathway in osteoblastic cells.28

Instead of relying on a single antioxidant effector, Nrf2 activation results in the coordinated upregulation of several antioxidant enzymes and cytoprotective genes, making it an attractive target for treatment of diabetic complications.29 Our in vitro studies showed that CGA attenuated ROS production, preserved NO levels, and decreased P22phox, P47phox, P67phox, and nitrotyrosine levels in HUVECs under HG conditions. Consistently, previous studies demonstrated that NADH/NADH oxidase can be
activated via upregulation of P22\textsuperscript{phox}, P47\textsuperscript{phox}, P67\textsuperscript{phox}, and represents the predominant source of ROS in the vasculature.\textsuperscript{30} To confirm the role of Nrf2 in HUVECs, we used an Nrf2 siRNA that substantially decreased the expression of Nrf2. We found that the protective effects of CGA were abolished by silencing of Nrf2. Studies of Nrf2\textsuperscript{−/−} and matched wild-type mice further confirmed these results. CGA pretreatment inhibited ROS generation and preserved NO levels in the endothelium of wild-type mice, but not Nrf2\textsuperscript{−/−} mice. Endothelium-dependent relaxation, but not endothelium-independent relaxation, was impaired under HG conditions in wild-type mice. Pretreatment with CGA preserved
endothelium-dependent relaxation in wild-type mice, but not Nrf2−/− mice.

Because db/db mice recapitulate key features of human type 2 diabetes,31 we used db/db mice to investigate the effects of dietary CGA on diabetes-related endothelial dysfunction. After 12 weeks of dietary CGA administration, db/db mice showed attenuated ROS production, increased NO levels, and improved endothelium-dependent relaxation of aortic rings. These improvements were associated with increased Nrf2 expression and decreased P22phox, P47phox, P67phox, and nitrotyrosine levels following CGA treatment.

In conclusion, our findings suggest that CGA prevents hyperglycemia-induced endothelial dysfunction via the Nrf2 pathway.

Authors' Contributions

PW, PZ: conception and design; DW, JH, JW, YY, SL, and WL: collection and/or assembly of data; DW, JH, XL, XD, PZ, PW: data analysis and interpretation, manuscript writing, and manuscript revision; DW, JH, JW, YY, SL, XL, WL, XD, PZ, PW: final approval of manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81970262 to P.W.), the Central Funds Guiding the Local Science and Technology Development of Sichuan Province (2020ZYD036 to P.W.), the Innovation Team Project Department of Education of Sichuan Province (18TD0030 to P.W.), and the Major Project of Education Department in Sichuan Province (16ZA0293 to X.L.).

ORCID iD

Peijian Wang https://orcid.org/0000-0002-3622-4538

References

1. De Rosa S, Arcidiacono B, Chiefari E, et al. Type 2 diabetes mellitus and cardiovascular disease: Genetic and epigenetic links. Front Endocrinol (Lausanne) 2018; 9: 2.
2. Kayama Y, Raaz U, Jagger A, et al. Diabetic cardiovascular disease induced by oxidative stress. Int J Mol Sci 2015; 16: 25234–25263.
3. Weber KS, Nowotny B, Strassburger K, et al. The role of markers of low-grade inflammation for the early time course of glycemic control, glucose disappearance rate, and beta-cell function in recently diagnosed type 1 and type 2 diabetes. Diabetes Care 2015; 38: 1758–1767.
4. Pei HF, Hou JN, Wei FP, et al. Melatonin attenuates postmyocardial infarction injury via increasing Tom70 expression. J Pineal Res 2017; 62.
5. Forstermann U, Xia N and Li H. Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. Circ Res 2017; 120: 713–735.
6. Wang D, Luo P, Wang Y, et al. Glucagon-like peptide-1 protects against cardiac microvascular injury in diabetes via a cAMP/PKA/Rho-dependent mechanism. Diabetes 2013; 62: 1697–1708.
7. Loboda A, Damulewicz M, Pyza E, et al. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: An evolutionarily conserved mechanism. Cell Mol Life Sci 2016; 73: 3221–3247.
8. Gallorini M, Petzel C, Bolay C, et al. Activation of the Nrf2-regulated antioxidant cell response inhibits HEMA-induced oxidative stress and supports cell viability. Biomaterials 2015; 56: 114–128.
9. Niture SK, Khatri R and Jaiswal AK. Regulation of Nrf2 – an update. Free Radic Biol Med 2014; 66: 36–44.
10. Akila P and Vennila L. Chlorogenic acid a dietary polyphenol attenuates isoproterenol induced myocardial oxidative stress in rat myocardium: An in vivo study. Biomed Pharmacother 2016; 84: 208–214.
11. Santana-Galvez J, Cisneros-Zevallos L and Jacobo-Velazquez DA. Chlorogenic acid: Recent advances on its dual role as a food
additive and a nutraceutical against metabolic syndrome. *Molecules* 2017; 22: 358.

12. Stefanello N, Schmatz R, Pereira LB, et al. Effects of chlorogenic acid, caffeine and coffee on components of the purinergic system of streptozotocin-induced diabetic rats. *J Nutr Biochem* 2016; 38: 145–153.

13. Akila P, Asaikumar L and Vennila L. Chlorogenic acid ameliorates isoproterenol-induced myocardial injury in rats by stabilizing mitochondrial and lysosomal enzymes. *Biomed Pharmacother* 2017; 85: 582–591.

14. Tom EN, Girard-Thernier C and Demougeot C. The Janus face of chlorogenic acid on vascular reactivity: A study on rat isolated vessels. *Phytotherapy* 2016; 23: 1037–1042.

15. Tao G, Kahr PC, Morikawa Y, et al. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature* 2016; 534: 119–123.

16. Sun J, Pu Y, Wang P, et al. TRPV1-mediated UCP2 upregulation ameliorates hyperglycemia-induced endothelial dysfunction. *Cardiostasis Diabetol* 2013; 12: 69.

17. Tian XY, Wong WT, Xu A, et al. Uncoupling protein-2 protects endothelial function in diet-induced obese mice. *Circ Res* 2012; 110: 1211–1216.

18. Wang F, Pu C, Zhou P, et al. Cinnamaldehyde prevents endothelial dysfunction induced by high glucose by activating Nrf2. *Cell Physiol Biochem* 2015; 36: 315–324.

19. Bao L, Li J, Zha D, et al. Chlorogenic acid prevents diabetic nephropathy by inhibiting oxidative stress and inflammation through modulation of the Nrf2/HO-1 and NF-kB pathways. *Int Immunopharmacol* 2018; 54: 245–253.

20. Gimbrone MA Jr and Garcia-Cardena G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ Res* 2016; 118: 620–636.

21. Schwartz SS, Epstein S, Corkey BE, et al. A unified pathophysiological construct of diabetes and its complications. *Trends Endocrinol Metab* 2017; 28: 645–655.

22. Tosovic J, Markovic S, Dimitrić Markovic JM, et al. Antioxidative mechanisms in chlorogenic acid. *Food Chem* 2017; 237: 390–398.

23. Ye HY, Li ZY, Zheng Y, et al. The attenuation of chlorogenic acid on oxidative stress for renal injury in streptozotocin-induced diabetic nephropathy rats. *Arch Pharm Res* 2016; 39: 989–997.

24. Shi A, Shi H, Wang Y, et al. Activation of Nrf2 pathway and inhibition of NLRP3 inflammasome activation contribute to the protective effect of chlorogenic acid on acute liver injury. *Int Immunopharmacol* 2018; 54: 125–130.

25. Clifford MN, Jagannath IB, Ludwig IA, et al. Chlorogenic acids and the acyl-quinic acids: Discovery, biosynthesis, bioavailability and bioactivity. *Nat Prod Rep* 2017; 34: 1391–1421.

26. Naveed M, Hejazi V, Abbas M, et al. Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed Pharmacother* 2018; 97: 67–74.

27. Chen B, Lu Y, Chen Y, et al. The role of Nrf2 in oxidative stress-induced endothelial injuries. *J Endocrinol* 2015; 225: R83–R99.

28. Han D, Gu X, Gao J, et al. Chlorogenic acid promotes the Nrf2/HO-1 anti-oxidative pathway by activating p21(Waf1/Cip1) to resist dexamethasone-induced apoptosis in osteoblastic cells. *Free Radic Biol Med* 2019; 137: 1–12.

29. Matzinger M, Fischhuber K and Heiss EH. Activation of Nrf2 signaling by natural products – can it alleviate diabetes? *Biotechnol Adv* 2018; 36: 1738–1767.

30. Shin JY, Sohn J and Park KH. Chlorogenic acid decreases retinal vascular hyperpermeability in diabetic rat model. *J Korean Med Sci* 2013; 28: 608–613.

31. Giesbertz P, Padberg I, Rein D, et al. Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. *Diabetologia* 2015; 58: 2133–2143.