Nrf2/HO-1-inductive Preventive Effect of 3,5-dicaffeoylquinic Acid, Antioxidant Compound from Green Coffee Beans, on Dimethylnitrosamine-induced Liver Fibrosis in Rats

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Abstract In this study, we investigated the hepatoprotective properties of 3,5-dicaffeoylquinic acid (DQA), a polyphenolic compound from green coffee beans, against dimethylnitrosamine (DMN)-induced hepatic fibrosis rat models. DQA, up to 10 μM concentration, did not exhibit any cytotoxic effect on Chang liver cells and Huh7 cells. DMN-treated rats showed typical biological symptoms of hepatic fibrosis, i.e., loss of body weight and pathological elevation of serum biomarkers for hepatic function. However, oral administration of DQA recovered body weight, and maintained serum biochemical markers such as albumin, bilirubin, ALP, ALT and AST in the normal range. Notably, histological and western assay proved DQA significantly alleviated collagen accumulation (α-smooth muscle actin, alpha 1 collagen type I) in liver tissue. The upregulated tissue expression of erythroid 2–related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) suggested DQA upregulated the representative antioxidant enzymes and ameliorated the progression of hepatic fibrosis in DMN-treated rats.

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1. Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$) are generated during oxygen metabolism in the body. ROS possess unpaired electrons in their structure and highly reactive with other biomolecules. The biological half-lives of ROS are extremely short, range from 10^{-9} to several seconds, but they are responsible for significant biological events in the body [1]. Certain levels of ROS are essentially required for the cellular redox system, but large amount of ROS that exceed the capacity of antioxidant systems cause cellular inflammatory reactions and tissue damage. Chronic exposure of ROS beyond cellular antioxidant capacity eventually leads to degenerative organ failure.

The liver is an organ vulnerable to ROS attacks. Especially, hepatic stellate cells, activated under chronic oxidative stress, start to synthesize collagen. The excessive deposition of collagen in hepatic parenchyma proceeds to irreversible liver fibrosis, hepatic cirrhosis, and sometimes primary liver cancer [2]. Viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) are invariably accompanied by liver fibrosis [3]. In most hepatic diseases that progress to liver fibrosis, ROS plays an initiative role. Therefore, ROS scavenging is considered a basic treatment option for the fibrosis-related hepatic diseases.

A self-defense antioxidant system in the body does not always cope with excessive oxidative stress, especially in chronic hepatic disease [4]. So far, many antioxidants have been screened for the rescue of fibrosis-related hepatic diseases. Most antioxidants are polyphenolic compounds extracted from herbal medicine [5]. Polyphenols efficiently scavenge overflowing ROS in hepatocytes, upregulate the function of oxidoreductase, and enhance the activities of multiple antioxidant enzymes. However, therapeutic efficacy of dietary polyphenols is

unreliable due to its stability, and sometime solubility problems. Most polyphenols are extensively metabolized after an oral administration. The oral bioavailability of most polyphenols is too low, less than 5%, so appropriate therapeutic blood levels cannot be achieved [6].

In this study, we investigated the anti-fibrotic effects of 3,5-dicaffeoylquinic acid (DQA) in a dimethylnitrosamine (DMN)-induced hepatic fibrosis rat models. DQA is a hydroxycinnamic acid derivative isolated from green coffee bean extract. Antioxidant and hepatic protective effects of coffee been extract have been reported before [7,8]. However, the prophylactic activity of DQA as a single extract compound from green coffee bean against liver fibrosis has not been reported yet. Furthermore, DQA is relatively stable in the body, and consequently shows the higher oral absorption rate enough to achieve therapeutic blood concentration [9] For the study, cytotoxicity of DQA was evaluated against the hepatic cell lines (Huh7 and Chang liver cells). Serum biochemical markers related with hepatic functions, body weight, and fibrous degeneration of liver tissue were observed after an oral administration of DQA against DMN-induced liver fibrosis rat models. Levels of antioxidant enzymes on the liver tissues were estimated to suggest the molecular mechanism of DQA.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-ethylenediaminetetraacetic acid (EDTA) disodium salt, FBS, penicillin, and streptomycin were purchased from Gibco BRL (Paisley, Scotland, UK). Dimethylnitrosamine (N-nitrosodimethylamine; DMN), 3,5-dicaffeoylquinic acid (DQA), and hydroxyproline were purchased from Sigma (St. Louis, MO, USA).

2.2. Measurement of Cellular Toxicity of DQA

Huh7 cells (human hepatoma cells) and Chang liver cells (human hepatocyte derived cells) were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM medium containing 10% fetal bovine serum (Gibco), and 1% penicillin/1% streptomycin. For cytotoxicity assays, cells were seeded into 96-well culture plates at $1x10^3$ cells/well in 0.2 ml of culture medium. After 24hrs of incubation at 37°C, media were replaced with 0.2 ml of different concentrations of DQA and further incubated for 72hrs. Cell ciabilities were measured using EZ-cytox assay kits (Daeil Lab Service, Chungcheongbuk-do, Korea). UV absorption at 450nm was measured using an Infinite M200 micro-plate reader (Tecan, Zürich, Switzerland).

2.3. DMN-induced Hepatic Fibrosis Modeling in Rats

All animal experiments were approved by Institutional Animal Care and Use Committees (IACUC) at INHA university following the National Institues of Health guidelines for laboratory animal care. Male Sprague-Dawley rats weighing 200-240 g were purchased from Orient-Bio Co. (Gyeonggi-do, Korea). Rats had unlimited access to chow and water and were housed in conventional cages at 25°C under a 12h dark/light cycle.

Twelve rats were divided into four groups. Control rats received the vehicle only. Fir the induction of hepatic fibrosis, DMN was intraperitoneally injected to rats at the dose of 1 ml (diluted 1:100 with 0.15 M sterile NaCl) per 100 g of body weight. The injections were given on three consecutive days of each week for a period of 4 weeks. DQA-treated groups were orally received 100 mg/kg of DQA three times per week for 4 weeks. At the end of the fourth week, rats were sacrificed under anesthesia. The liver tissues were recovered and weighted. Blood samples were collected by cardiac puncture, and serum samples were recovered by centrifuging blood samples at 3,000 rpm for 15 min at 4°C. Liver specimens were immediately fixed in 10% neutral buffered formaldehyde for histochemical study. Serum and liver tissue samples were stored at -80°C for other studies.

2.4. Measurements of Serum Enzyme Biomarkers

Serum samples were separated from whole blood by centrifugation at 1800 g (RCF) for 15 min at 4°C. Serum alanine amino transferase (ALT), aspartate amino transferase (AST), total protein, alkaline phosphatase (ALP), total bilirubin (T-bilirubin), direct bilirubin (D-bilirubin), and albumin were analyzed by Green Cross Labcell (Gyeonggi-do, Korea).

2.5. Measurements of Hepatic Malondialdehyde Levels

Malondialdehyde (MDA) a major byproduct of lipid peroxidation resulting from oxidative stress. MDA levels in liver tissues were determined using a Bioxytech MDA-586 Assay Kit (OxisResearchTM, Portland, OR). Briefly, liver tissues were homogenized after mixing with butylated hydroxytoluene. The supernatants were collected, and then reacted with N-methyl-2-phenylindole. Amounts of carbocyanine dye produced by reaction between MDA and N-methyl-2-phenylindole were determined by measuring absorbance at 586 nm (Infinite M200, Tecan, Switzerland). Protein concentrations were determined using Protein Assay Kits (Pierce Biotechnology,Waltham, MA). MDA levels were normalized versus tissue protein levels (nmol/mg) [10]

2.6. Measurement of Hepatic Hydroxyproline Levels

Total amounts of collagen in liver tissues were determined by measuring tissue hydroxyproline (the major amino acid component of collagen) contents, as previously described [11]. Briefly, liver tissues were homogenized with 1.0 ml of 6 N HCl. The homogenized liver samples were then autoclaved at 120°C for 20 minutes to hydrolyze collagen into its component amino acids. Samples were then evaporated to dryness in a vacuum oven (65°C for 2 days). The dried pellets obtained were

resuspended in 1.0 ml of 1.0 mM HCl, and vortexed for 1 minute. Standards or samples (50 µl) were then transferred to 1.5 ml centrifuge tubes, mixed with 450 µl of chloramine T, vortexed for 30 secs, allowed to stand at room temperature for 25 minutes, and then reacted with Ehrlich's reagent (500 µl) for 20 minutes at 60°C. Absorptions of samples were measured using a spectrophotometer at 558 nm. Trans-4-hydroxy-L-proline was used as the standard.

2.7. RT-PCR for Estimation of Fibrosis-related Gene

RNA was isolated from frozen rat liver tissues using Trizol® solution (Invitrogen, Carlsbad, CA). Single-strand cDNA was prepared from 1 µg of total RNA using a reverse transcription system (Promega, Madison, WI), according to the manufacturer's instructions. cDNA was subsequently amplified by PCR in a reaction volume of 25 µl containing 1xPCR reaction buffer, 200 µM dNTPs, 0.5 pmol specific primer for each gene, and 1 unit of Taq DNA polymerase (TaKaRa Biotech, Shiga, Japan). GAPDH was used as the reference gene for DNA synthesis and PCR amplification. The primer sets were purchased from Thermo Fisher Scientific (Waltham, MA, USA).
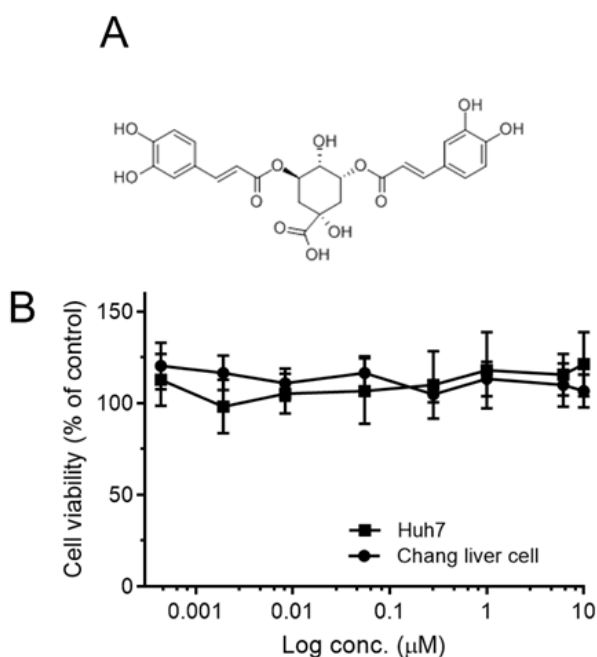
Figure 1. Cellular toxicities of 3,5-dicaffeoylquinic acid (DQA) on hepatic cells. (A) Chemical structure of 3,5-dicaffeoylquinic acid (DQA) [(3R,5R)-3,5-bis[[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy]-1,4-dihydroxycyclohexane-1-carboxylic acid]. (B) Cytotoxicity assays were performed using WST-1 assays to evaluate the dose-dependently effects of DQA on human hepatocytes (Chang liver cells) and human hepatoma cells (Huh7 cells). Results are presented as the mean±SD of 3 independent experiments

2.8. Western Blot Analysis of Hepatic Tissues

Liver tissue was homogenized in a radioactive immunoprecipitation assay buffer containing a protease inhibitor, and the tissue homogenate was centrifuged at 15,000 rpm for 15 minutes to obtain protein. Protein concentrations were determined using a bicinchoninic acid-based method [12]. Liver proteins (40 µg) were separated by 2D 10% SDS-PAGE, and the resolved proteins were transferred onto 0.2 µm nitrocellulose membranes (Schleicher & Schuell, Middlesex, UK). Blots were blocked in 5% skim milk in PBS, incubated with primary antibodies, conjugated with secondary antibodies, and stained with chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). The primary antibodies used were mouse monoclonal anti α-SMA (Abcam, Cambridge, UK), rabbit polyclonal anti-Nrf2, anti HO-1 and β-actin (Santa Cruz Biotechnology, Dallas, TX). Secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

2.9. Histological Study

Liver specimens were fixed in 10% buffered formaldehyde for 18 to 24 hours, embedded in paraffin, and sectioned. After deparaffinization and dehydration, specimens were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT) for microscopic observation (Axio Vert, Carl Zeiss AG, Oberkochen, Germany). Immunohistochemical staining for estimation of α-SMA in tissues was performed. The primary antibodies used were anti α-SMA (Santa Cruz Biotechnology, Dallas, TX). Alexa Fluor® 555-conjugated secondary antibodies were used for fluorescence microscopy (Carl Zeiss AG, Oberkochen, Germany).

2.10. Statistical Analysis

The student t-test was used to determine the significances of differences. Results are presented as mean± standard deviation (STD).

3. Results and Discussion

3.1. Cytotoxicity of Antioxidant DQA

DQA, one of major components in green coffee bean extract, is chemically composed of two caffeic acid molecules bridged by quinic acid via ester bonds (Figure 1A) [7]. First, we observed cellular toxicity of DQA in Huh7 and Chang liver cells. As shown in Figure 1B, DQA did not show any substantial cytotoxicity against the human hepatoma cells or Chang liver cells at concentrations of ≤10 µg/mL.

Jho et al. reported DQA protected HepG2 cells from tert-butyl hydroperoxide (t-BHP)-induced oxidative cell death. They proved cell survival after t-BHP treatment reduced to < 10% but reduced to only 70% when t-BHP was co-treated with 80 µM of DQA [13]. However, they only observed the antioxidant effect of DQA on HepG2.

3.2. Effect of DQA on Body Weight and Serum Enzyme Levels

For the study, DQA was orally administered three times per week for 4 weeks while DMN was administered i.p. for 3 consecutive days per week during the same 4 weeks. Figure 2 shows slight increase of body weight in DQA

alone group and DQA-DMN co-treated group when compared with non-treat control and DMN-treated group, respectively. Liver weight displayed the same patterns with body weight change. Relative liver weight (liver weight/final body weight) in DMN group and DMN+DQA co-treated group were around 2.95% and 3.39%, respectively (P<0.1).
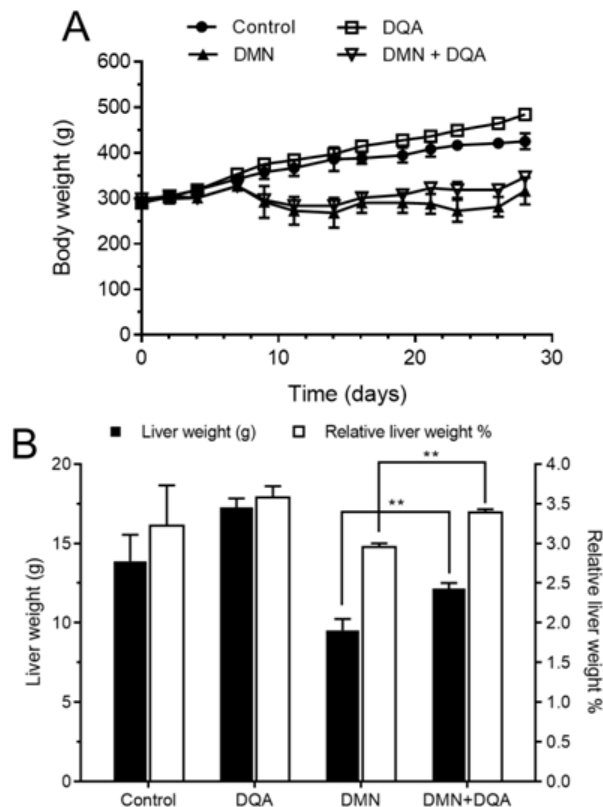
Figure 2. Effects of 3,5-dicaffeoylquinic acid (DQA) on the body and liver weights of rats treated with DMN. (A) DQA was orally administered three times per week for 4 weeks while DMN was administered i p. for 3 consecutive days per week during the same 4 weeks. Body weights were measured during the 4-week treatment period. (B) Whole liver weights and relative liver weights (expressed as percentages of body weights) were evaluated after the 4 weeks of treatment. *P<0.1 and **P<0.05; DMN group versus DMN+DQA group cells, and further systematic studies are needed for the proofs of in vivo antioxidant effect of DQA

In this experiment, we observed the safety of DQA through physical and serum biochemical observations of rats during 4 weeks of oral feeding of DQA. Lee et al. studied dietary feeding effect of caffeoylquinic acid-rich extract of Aster glehni on ApoE KO Mice. Experimental results showed dose-dependent decrease of body weight and abdominal adipose tissue weights [14]. However, our data showed slight increase of body weight in DQA group and DQA+DMN group when compared with non-treat control and DMN-treated group, respectively (Figure 2). These results imply DQA helps the recovery of body weight, as well as liver weight against DMN-induced liver injury.The protective effect of DQA on DMN-induced liver injury was evaluated by determining the levels of serum ALT, AST, ALP, T-bilirubin, and albumin. As shown in Figure 3, DMN significantly increased the levels of ALT, AST, ALP, and T-bilirubin as compared with the control group. However, these levels recovered when treated with DQA. Conversely, plasma protein levels (albumin) decreased after treatment with DMN, and recovered when treated with DQA.

3.3. Effect of DQA on Fibrotic Deformation of Hepatic Tissues

Rat livers stained with H&E and Masson's trichrome (MT) showed extensive collagen accumulation the DMN group (Figure 4., yellow arrows). Control livers showed a normal lobular architecture with central veins and radiating hepatic cords, whereas DMN-treated rat liver showed a distinct histologic pattern of fibrotic septa, the incorporation of degenerated hepatocytes into pseudo-lobules, and higher collagen contents than control livers (Figure 4A, Control vs. DMN). DMN+DQA treatment markedly alleviated the degree of liver fibrosis and significantly reduced the collagen deposition (Figure 4A, DMN vs. DMN+DQA).

Total collagen in livers was estimated by measuring liver hydroxyproline levels (a quantitative marker of fibrosis). Hydroxyproline content of the DMN group were all higher than those of the control group (Figure 4C), and this DMN-mediated increase in hepatic hydroxyproline was suppressed in the DMN+DQA group. This result suggested DQA attenuated DMN-induced hepatic collagen deposition.

MDA contents in liver tissues in the DMN group were significantly higher than those of non-treatment control group (Figure 4D, control vs. DMN), but co-treatment with DQA suppressed these DMN-induced MDA (Figure 4D, DMN vs. DMN+DQA).

The elevated levels of MDA and hydroxyproline in hepatic tissues suggest that oxidative stress is the main cause of the hepatic deformation. Administration of DMN induces oxidative stress, and the generated ROS reacts with polyunsaturated lipids to form MDA which is a toxic product of lipid peroxidation. Finally, MDA causes fibrotic deformation of liver tissues [15,16].

We observed rats treated with DMN showed elevated serum biochemical markers (AST, ALT and ALP) (Figure 3). Furthermore, our histologic study revealed hepatic lobes in the DMN-treated group showed severe hepatic fibrosis from fibril deposition and collagen accumulation (Figure 4).

3.4. Effect of DQA on α-SMA, Type 1 collagen, Nrf2 and HO-1

The effects of DQA on the expressions of hepatic fibrosis-related factors (i.e., Type 1 collagen and α-SMA) and antioxidant enzymes (i.e., Nrf2 and HO-1) in liver tissue were evaluated by RT-PCR, western blot and immunofluorescence analyses (Figure 4B and Figure 5).

Fragments specific for alpha smooth muscle actin (α-SMA) and alpha 1 collagen type I (COL1A1) were amplified by RT-PCR (Figure 5A). The results of the densitometric analysis were normalized against the corresponding GAPDH transcript. Hepatic expressions of α-SMA and COL1A1 in the DMN group were 170.3% and 121.1% higher, respectively, than those in controls. However, co-treatment with a DQA (100 mg/kg) showed a decrease of 12.6% and 97.2%, respectively, in α-SMA and COL1A1 of mRNA expression in comparison

with the DMN group (p<0.001). The relative mRNA expressions of Nrf2 increased from 9.37 % in DMN group to 64.9 % in DMN+DQA group. These results clearly showed that the mRNA expressions of COL1A1 and α-SMA were elevated after DMN injection while the oral administration of DQA blocked the elevation of those liver fibrosis-related markers (Figure 5A and Figure 5B). Immunofluorescence analyses also proved DMN+DQA treatment significantly suppressed the expression levels of α-SMA when compared with α-SMA expression level of with DMN group (Figure 4B, DMN vs. DMN+DQA).
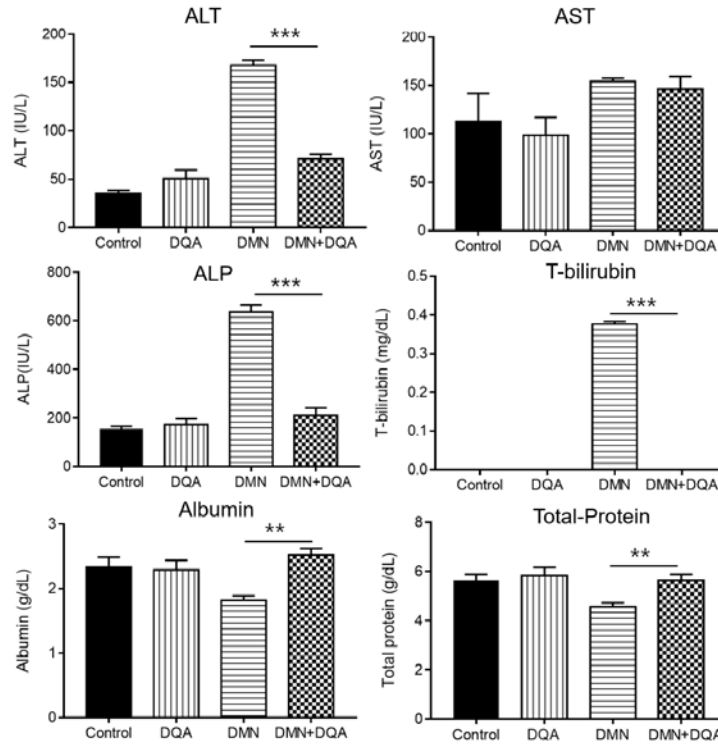
Figure 3. Effects of 3,5-dicaffeoylquinic acid (DQA) on blood biochemical factors in our rat model of hepatic fibrosis. Serum albumin, total protein, ALT, AST, ALP, and total bilirubin were measured after the 4 weeks of treatment. Control, treatment naive control; DQA, DQA group; DMN, DMN group; DMN+DQA, DMN and DQA co-treated group. **P<0.05 and *** P<0.01; DMN group versus DMN+DQA group
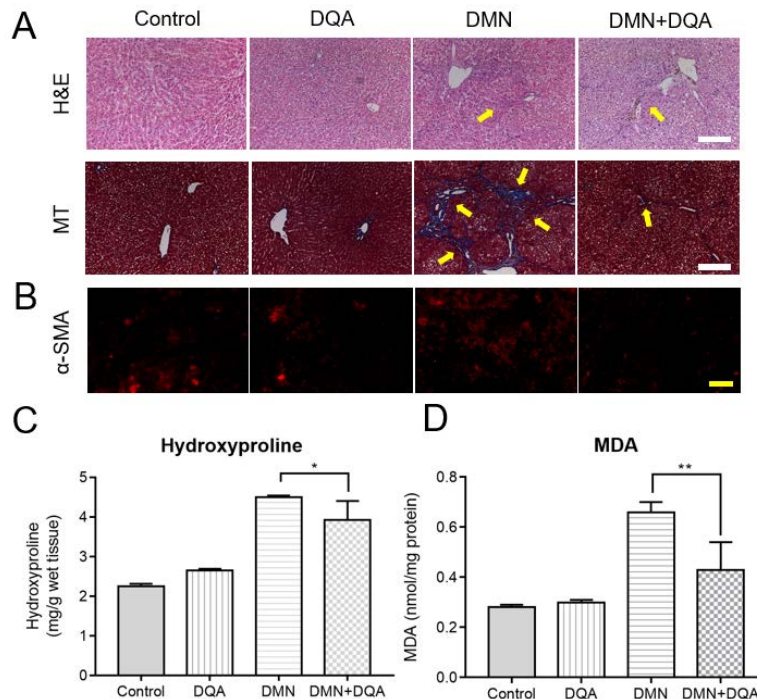
Figure 4. Effects of 3,5-dicaffeoylquinic acid (DQA) on hepatic tissue histology, oxidative factor dispositions, and on fibrosis-related factors. (A) Histopathological examination of rat liver tissues. Rat liver sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT). (B) Immuno-histological observation of α-SMA in rat liver tissues. (C and D) Hepatic levels of malondialdehyde (MDA) and hydroxyproline (HP) were measured. Results are presented as the mean±SD of 3 independent experiments. White and yellow bars represent 200 μm and 100 μm, respectively. *P<0.1 and **P<0.05; DMN group versus DMN+DQA group
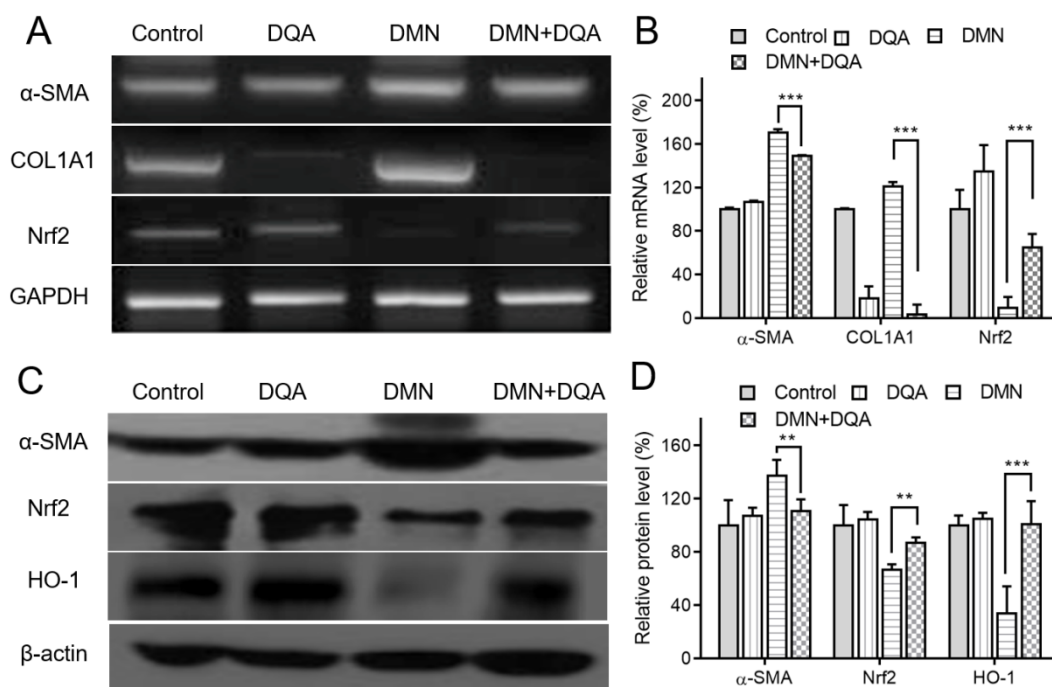
Figure 5. Effects of 3,5-dicaffeoylquinic acid (DQA) on collagen disposition and antioxidant enzyme levels in hepatic tissues. (A) The mRNA expression levels of collagen I (COL1A1), the myofibroblastic myogenic marker; α-SMA and antioxidant enzyme; Nrf2 were assessed by RT-PCR. (B) Protein levels of Nrf2, HO-1and α-SMA were determined by western blotting. **P<0.05 and *** P<0.01; DMN group versus DMN+DQA group

Protein expressions of α-SMA, Nrf2 and HO-1 in hepatic fibrotic septa were also observed. In whole liver lysates, DQA decreased the expression of α-SMA and increased that of Nrf2. α-SMA protein levels were 115% higher in the DMN group than in controls (P<0.1) and were 16% lower in the DMN+DQA group than in the DMN group (p<0.05) (Figure 5C and 5D). Nrf2 protein levels were increased by DQA treatment. Nrf2 level in the DMN+DQA group was 116% higher (p<0.1) than in the DMN group. Accordingly, the HO-1 level in the DMN + DQA group was increased to 311.0% compared to the DMN group. The induction mechanism of Nrf2 by DQA is not fully proved in this study. However, it is generally reported that Nrf2 up-regulates antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase (SOD), and glutathione S-transferase family, and protect ROS-driven lipid peroxidation and resulting fibrosis [17,18]. We are also supposing the antioxidant effect of DQA follows the same mechanism.

3.5. Polyphenols and DQA for antioxidant effect

Polyphenols are classified as ubiquitous secondary plant metabolites which possess one or more hydroxyl groups with single or multiple aromatic rings. Polyphenols upregulates the activities of multiple antioxidant enzymes, neutralizes the overflowing ROS in the cells and thus display antioxidant activities. However, dietary polyphenols undergo massive degradation after an oral administration. Its therapeutic activity as an antioxidant is always under the question [6,19]. For example, bioavailability of gallic acid and isoflavones is only around 5%, and other flavan-3-ol (a catechin), flavanone, and quercetin glucoside shows lower than 5% of bioavailability [9]. In addition, some polyphenols, such as curcumin, have poor solubility which severely limits their oral absorptions [20,21]. The therapeutic efficacies of proanthocyanidins, galloylated tea catechins, and anthocyanins are also limited by their poor oral absorptions [22].

Green coffee extracts have many beneficial effects on human health, for example, they have been reported to exhibit antihypertensive and peripheral vasodilatory effects [23,24,25], to suppress body weight gain and fat deposition [26,27], and to modulate glucose metabolism in man [28]. However, the therapeutic efficacy of a single compound from green coffee bean extract has not been thoroughly studied yet. DQA is a relatively stable compound, and consequently its oral absorption is greater than other polyphenols [9] Caffeic acid, a major component of DQA, was reported to be highly stable enough to maintain its biological effects after systemic circulation (Figure 1A) [29]. Farah et al reported caffeoylquinic acids, the major compound of green coffee extract, shows higher oral bioavailability and extended blood circulation time [30]. They reported the apparent bioavailability of caffeoylquinic acid was around 33%. Interestingly, the reported Tmax of caffeoylquinic acid was between 0.5 and 8 hours and much longer than other phenolic antioxidants. Therefore, DQA probably show more reliable preventive effects on hepatic fibrosis induced by chronic ROS exposures. A major implication of our study is that DQA, which is reported be relatively, stable after an oral administration, modulates the serum biochemical and hepatic oxidative stress markers as well as the histological deformations of hepatic tissues without any adverse effects on the rat models (Figure 6).
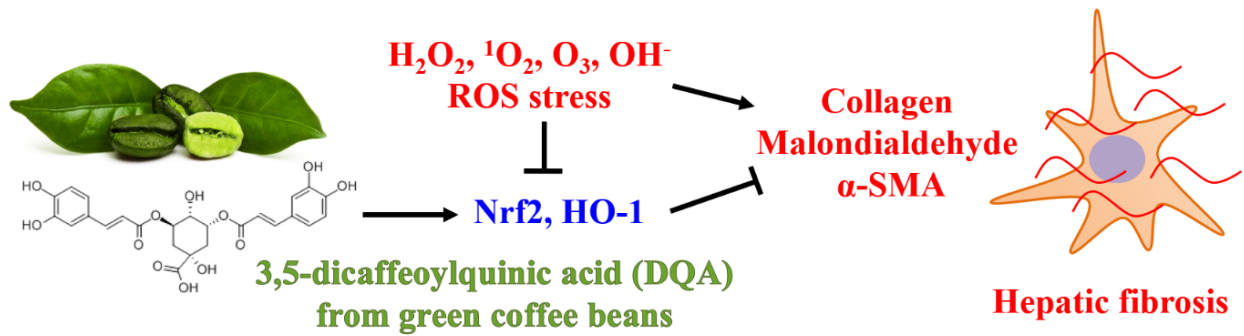
Figure 6. Nrf2/HO-1-inductive preventive effect of 3,5-dicaffeoylquinic acid, an antioxidant compound from green coffee beans, on oxidative stress-related liver fibrosis

4. Conclusion

In this study, we proved 3,5-dicaffeoylquinic acid (DQA) has a preventive effect on DMN-induced hepatic fibrosis models in rats. Although further studies are required to determine the mechanism responsible for the therapeutic effect of DQA, the present study does demonstrate DQA can attenuate the progress of hepatic fibrosis in DMN-induced rat models, potentially by activating the Nrf2 pathway.

Acknowledgements

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