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Anti-fibrotic effects of the anthocyanins isolated from the purple-fleshed sweet potato on hepatic fibrosis induced by dimethylnitrosamine administration in rats

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ABSTRACT

In current study, we investigated the protective effects of the anthocyanin fraction (AF) obtained from the purple-fleshed sweet potato on hepatic fibrosis induced by dimethylnitrosamine (DMN) administration in rats. Treatment with DMN for 4 weeks produced marked liver fibrosis as assessed by increased serum alanine aminotransferase and aspartate aminotransferase activity and hepatic collagen content. These increases were inhibited by treatment with AF prior to the administration of DMN. In addition, AF inhibited DMN-induced reductions in rat body and liver weights in a dose-dependent manner. Histopathological evaluation of the rat livers revealed that AF reduced the incidence of hepatic fibrosis lesions and inhibited DMN-induced increases in α-smooth muscle actin (α-SMA) and collagen type I and III expression levels. AF also decreased DMN-induced expression levels platelet-derived growth factor receptors-beta, tumor necrosis factor-alpha and transforming growth factor-beta. This study demonstrates that AF administration can effectively improve liver fibrosis caused by DMN, and may be used as a therapeutic option and preventive measure against hepatic fibrosis.

1. Introduction

Hepatic fibrosis is caused by a variety of pathological factors, including alcohol consumption, drug abuse, autoimmune disease, metabolic disease, constant cholestasis, and hepatic viruses (Friedman, 2000). Hepatic fibrosis, a response to chronic liver injury, results from the excessive deposition of extracellular matrix (ECM) proteins and can ultimately lead to cirrhosis of the liver (Hsiang et al., 2005). Hepatic stellate cells (HSCs) are pericytes found in the perisinusoidal space of the liver (a small area between the sinusoids and hepatocytes). In response to liver injury HSCs undergo rapid activation, which leads to functional and morphological changes. Activated HSCs are proliferative and fibrogenic and cause the accumulation of ECM proteins, including transforming growth factor-beta (TGF-β), alpha-smooth muscle actin (α-SMA) and collagen (Friedman, 2008; Kisseleva and Brenner, 2008; Henderson and Iredale, 2007; Wallace et al., 2008).

During hepatic fibrogenesis, HSCs are activated by reactive oxygen species (ROS), growth factors, and profibrogenic cytokines released from damaged hepatocytes and Kupffer cells (Kisseleva and Brenner, 2007), and their cognate receptors are associated with this transition. Among these factors, autocrine and paracrine signaling via platelet-derived growth factor (PDGF), a potent ligand for PDGF receptors (PDGFRs), stimulates HSC growth and proliferation (Lotersztajn et al., 2005; Pinzani and Marra, 2001). Also, it has been reported that hepatocellular injury mediates inflammation response by tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) and enhances the proliferation of cultured stellate cells and their collagen synthesis (Casini et al., 1994; Hierholzer et al., 1998; Ramadori and Armbrust, 2001).

Dimethylnitrosamine (DMN) leads liver fibrogenesis and subsequent cirrhosis. DMN exhibits a potent hepatotoxicant through metabolic activation by cytochrome P450 2E1 (CYP2E1) in animal experimental models (George et al., 2001). DMN-induced hepatic fibrosis model to reproduce most of the features observed during human liver fibrosis, such as ascites, nodular regeneration, overproduction of extracellular matrix including collagen and histopathological changes (Bataller and Brenner, 2005). It is a valuable animal model for studying mechanisms of hepatic fibrosis, and may provide a model for the rapid screening of anti-fibrotic agents.

Anthocyanins, a class of naturally presenting polyphenol compounds, are widely distributed in fruits, beans, cereals, and vegetables. In animal models, the biological activity of anthocyanins includes powerful antioxidant effects (Shih et al., 2007), anti-inflammatory effects (Karlsen et al., 2007), and anti-tumor

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properties through the stalling of the growth of pre-malignant cells (Shih et al., 2005). Anthocyanins also help to prevent obesity, hyperglycemia (Tsuda et al., 2003), and asthma (Park et al., 2007). The component part of vegetables and fruits containing abundant plant polyphenols is associated with a low risk of life-style-related diseases, such as cardiovascular disease (Hertog et al., 1993; Keli et al., 1996; Aviram and Fuhrman, 1998; Arts and Hollman, 2005). Recently, purple sweet potato (*Ipomoea batatas*) has aroused extensive attention because of its unique color, nutrition and its role in health care (Lila, 2004). There is a high content of anthocyanin pigments in the tuber of some purple sweet potato cultivars. The anthocyanins from purple sweet potato are more stable than the pigments of strawberry, red cabbage, perilla and other plants. So purple sweet potatoes have been regarded as a good source of stable anthocyanins as a food colorant and purple sweet potato color could be recognized as a physiologically functional food factor. Anthocyanins of purple sweet potato possess biological functions such as scavenging free radicals, anti-mutagenicity, anti-carcinogen activity and anti-hypertensive effects (Ahmed et al., 2010). We previously reported that the anthocyanin fraction (AF) obtained from the purple-fleshed sweet potato has a potent hepatoprotective effect in acetaminophen (APAP)-induced hepatic damage mouse model (Choi et al., 2009). AF upregulated the activities of antioxidants such as glutathione and glutathione S-transferase, and acts as a free radical scavenger. Also, AF inhibited the APAP-induced hepatotoxicity through blocking of CYP2E1-mediated APAP bioactivation. However, the anti-fibrotic effects of AF have not reported. In current study, we investigated the anti-fibrotic effects of AF on hepatic fibrosis induced by DMN administration in rats.

2. Materials and methods

2.1. Preparation of AF

AF was purified from the aqueous extract of whole bodies of purple-fleshed sweet potato supplied by the Ji San Food Co. (Hamyang, Korea). AF was prepared as described elsewhere (Lee et al., 2000) and their compositions were previously published. The compositions of the purple-fleshed sweet potato of anthocyanin were cyanidin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride, pelargonidin-3-O-glucoside chloride, and peonidine-3-O-glucoside chloride (Goda et al., 1997). Briefly, uniformly sized tubers without defects were washed, peeled, diced into 0.5-cm cubes, and freeze-dried. Freeze-dried tuber samples (0.5 g) were homogenized in 15 mL ethanol/water (85:15 vol/vol) using an Ultra Turrax (Divtech Equipment Co., Cincinnati, OH), Tissumizer (30,000 rpm) and stored for 12 h at −20 °C. Supernatants obtained by centrifugation were concentrated, diluted to 5 mL using 0.01% aqueous HCl (whole extract), and passed through C-18 Sep-Pak cartridges (Waters, Milford, MA) preconditioned with 0.01% acidified methanol to absorb anthocyanins (Lee et al., 1997). Anthocyanins were obtained by eluting columns with 0.01% methanolic HCl, concentrating eluates (under a nitrogen flow), and reconstituting with either alcohol or dimethyl sulfoxide. The yield of dried residue corresponded to 2.5% of the original dry whole body weight. This was powdered in a grinder, passed through a 40-mesh sieve, and stored at −20 °C until use.

2.2. Animals and treatment

Five-week-old male Sprague–Dawley (SD) rats were obtained from Daehan Biolink (Chungbuk, Korea). The animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water and were maintained under specific pathogen-free conditions. Animals were acclimatized to the temperature (22 ± 2 °C) and humidity (55 ± 5%) of controlled rooms with a 12-h light/dark cycle for at least 1 week prior to experimentation. All animal experiments were performed according to the rules and regulations of the Animal Ethics Committee, Chosun University.

The rats were divided into six groups. To induce hepatic fibrosis, we administered DMN (Sigma Chemical Co., St. Louis, MO) dissolved in sterile saline (10 mg/kg body weight) as an intraperitoneal injection three times per week for 4 weeks. AF was dissolved in saline. Rats were intragastrically administered 50, 100, and 200 mg/kg of AF per day six times per week for 4 weeks. The control and DMN-treated groups were administered saline (intragastrically) without drug administration. The animals were sacrificed on day 29 (Fig. 1). Each group consisted of five rats. Livers were excised, were weighed, and underwent histopathological examination and determination of collagen content using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland).

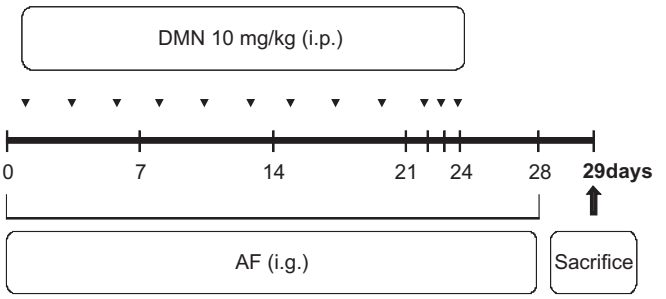

Fig. 1. Schematic diagram of the experimental procedure. Rats were divided into six groups. To induce hepatic fibrosis, we administered DMN dissolved in sterile saline (10 mg/kg body weight) by intraperitoneal (i.p.) injection three times per week for 4 weeks. AF was dissolved in saline. Rats were intragastrically (i.g.) administered 50, 100, and 200 mg/kg/day of AF six times per week for 4 weeks. The control and DMN-treated groups were administered saline alone (i.g.) without drug. The animals were sacrificed on day 29. Each group consisted of five rats.

2.3. Hepatotoxicity studies

To assess hepatotoxicity, we measured the serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using spectrophotometric diagnostic kits (Sigma Chemical Co., St. Louis, MO, USA).

2.4. Histological examinations

The left lateral lobe of the liver was sliced, and tissue slices were fixed in 10% buffered-neutral formalin for 24 h. The fixed liver tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Sections 5 μm in thickness were subjected to hematoxylin and eosin and Masson's trichrome staining prior to examination (Vyberg et al., 1987). An arbitrary scope was given to each microscopic field viewed at a magnification of 100×. A minimum of 10 fields were scored per liver slice. The extent of fibrosis was graded as 0, no increase; 1, slight increase; 2, moderate increase; 3, distinct increase; or 4, severe increase. The extent of periportal bridging, intralobular degeneration, portal inflammation, and fibrosis was also graded according to Knodell's scoring method (Moragas et al., 1998).

2.5. Collagen content

The right lobe of the liver (0.2 g) was homogenated with 0.5 M acetic acid containing 1 mg pepsin (at a concentration of 10 mg tissue/10 ml acetic acid solution). The resulting mixture was then incubated for 24 h at 4 °C with stirring. Liver collagen content was determined by assaying total soluble collagen using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland) according to the manufacturer's instructions. Acid soluble type I collagen supplied with the kit was used to generate a standard curve.

2.6. Semi-quantitative RT-PCR

Total RNA was extracted from frozen liver samples with RNAiso reagent (Takara, Kyoto, Japan) according to manufacturer's protocol and stored at −80 °C until use. Then 0.5 μg RNA was used for reverse transcription and amplified by polymerase chain reaction (PCR) using the access RT-PCR system Takara thermal cycler (TaKaRa, Seoul, Korea). The PCR amplification protocol was 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s. The termination cycle included a prolonged extension at 72 °C for 7 min. Amplified products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. The coding sequences of the genes are presented in Table 1.

2.7. Western blotting

To analyze protein expression, we homogenized liver tissues in a Potter–Elvehjem homogenizer with four volumes (w/v) of 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.01 mM phenylmethoxysulfonyl fluoride. Then 50–150 μg protein was routinely resolved by SDS–PAGE, transferred to a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with the appropriate primary and secondary antibodies. PDGFR-β, COL1A1, COL3A1, and β-actin (C4) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody was a horseradish peroxidase-coupled anti-rabbit or mouse IgG (Beverly, MA, USA). Anti-α-SMA antibody was purchased from Dako (Glostrup, Denmark). Membranes were probed with an ECL western blot detection system according to the manufacturer's instructions.

Table 1
Semi-quantitative PCR primer.

Gene	Primer sequence (5′–3′)	Tm (°C)	Cycles
α-SMA	F: GCTCTGTAAGGCGGGCTTTG R: ACGAAGGAATAGCCACGCTCA	56	28
Collagen type I	F: GGTAACGATGGTGCTGTCGG R: GGGACCTTGAACTCCAGCAG	56	29
Collagen type III	F: AGATCATGTCTTCACTCAAGTC R: TTTACATTGCCATTGGCCTGA	56	30
TNF-α	F: GCCAATGGCATGGATCTCAAAG R: CAGAGCAATGACTCCAAAGT	53	35
TGF-β1	F: TGGCGTTACCTTGGTAACC R: GGTGTTGAGCCCTTTCCAG	60	40
β-Actin	F: GCCATGTACGTAGCCATCCA R: GAACCGCTCATTGCCGATAG	55	30

2.8. Statistical analysis

All experiments were performed three times. Results are expressed as means ± SEM. Statistical significance was determined by a one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test. Significance was set at $P < 0.05$.

3. Results

3.1. AF reduces DMN-induced hepatotoxicity in rat liver

The liver uses a variety of transaminases to synthesize and break down amino acids and to allow for the interconversion of energy storage molecules. When the liver becomes damaged, the concentration of transaminases in the serum increases because of the increased permeability of the hepatocyte cell membrane (Luo et al., 1998). We thus used the serum levels of two transaminases, ALT and AST, as an indicator of liver injury. As shown in Fig. 2, serum ALT and AST activity increased in the DMN-treated group compared with the control group. Concurrent administration of AF to the DMN group significantly decreased both DMN-induced serum ALT and AST activity (Fig. 2A and B). The administration of AF (200 mg/kg body weight) alone did not influence the body weight or the relative liver weight of rats. However, the body and liver weights of the DMN-treated group were lower than those of the control group, and the rats had a sickened appearance and moved less vigorously. The mean body weight of rat in the DMN-treated group was approximately 60% that of the control group (Fig. 2C). However, co-administration of AF improved the decrease in body weight of those rats treated with DMN. A significant increase in the relative liver weight was detected in DMN-treated rats (Fig. 2D). AF treatment prevented the increase of the relative liver weight induced by DMN.

3.2. AF reduces DMN-induced fibrosis in rat liver

The rat livers treated with DMN for 4 weeks developed hepatic fibrosis. Histological analysis by HE and Masson's trichrome staining showed distorted tissue architecture with bundles of collagen surrounding the lobules as well as large fibrous septa (Fig. 3A and B). Co-administration of AF significantly attenuated the deposition of collagen fibers. In the DMN group, 5-rats presented liver fibrosis of score-2. In contrast, in the high-dose AF groups, there was 5-rats showed liver fibrosis of score-0.8 (Fig. 3C). These results suggest that AF can reduce DMN-induced hepatotoxicity.

3.3. AF reduces DMN-induced expression of α-SMA and collagen types I and III

Transactivation of quiescent stellate cells contributes to hepatic fibrosis (Toyoki et al., 1998). Activated HSCs are associated with cell proliferation and the accumulation of ECM proteins, including α-SMA and collagen type I and III. We therefore evaluated the

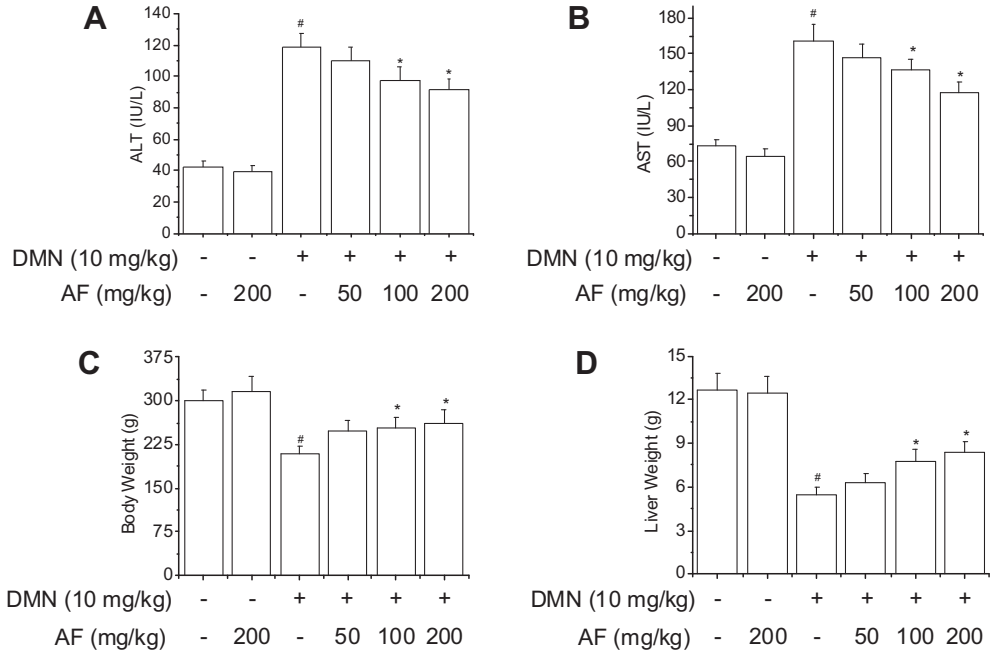

Fig. 2. Effects of AF on DMN-induced hepatotoxicity and body and liver weight loss. Hepatotoxicity was determined by quantifying the serum activity of (A) ALT and (B) AST and the weights of the (C) bodies and (D) livers of fibrotic rats. Results are the mean ± SEM for five rats in each group. Results were obtained from three independent experiments. ($^{\#}P < 0.05$) significantly different from the control group. ($^{*}P < 0.05$) significantly different from the DMN-treated group.

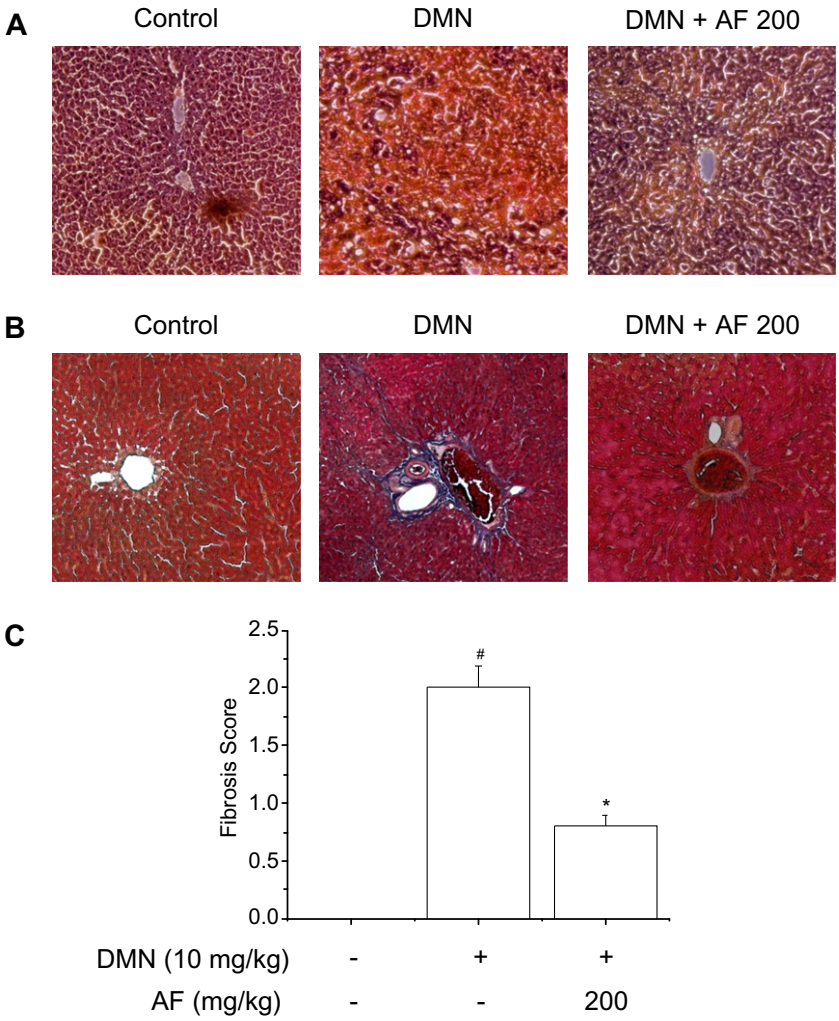

Fig. 3. Effects of AF on histopathological changes and hepatic collagen content by DMN. Liver tissues were collected and fixed with 10% formaldehyde. Thin sections (5 μm) were cut and stained with (A) hematoxylin and eosin and (B) Masson's trichrome. Liver tissues were obtained from rats administrated saline (Control), DMN-treated rats administrated saline (DMN), and DMN-treated rats administrated AF 200 mg/kg (DMN + AF 200). (C) Fibrosis scores were evaluated in the liver sections stained with Masson's trichrome in a blinded fashion. The extent of fibrosis was graded as described in Section 2. Results are the mean ± SEM for five rats in each group. Results were obtained from three independent experiments. ($^{\#}P < 0.05$) significantly different from the control group. ($^{*}P < 0.05$) significantly different from the DMN-treated group.

effects of AF on the levels of α-SMA and collagen type I and III expression in liver tissue as determined by semi-quantitative RT-PCR and western blotting analysis. As shown in Fig. 4, the expression of α-SMA and collagen types I and III were increased in DMN-treated group compared with control group, consistent with DMN-induced hepatotoxicity. Levels of α-SMA and collagen types I and III decreased compared with those of the DMN-treated group when AF was administrated (Fig. 4A and B). We further measured hepatic collagen content as an indicator of hepatic collagen accumulation. Hepatic collagen content was significantly elevated in the DMN-treated group compared with the control group. However, the AF concurrent administration group showed significantly decreased hepatic collagen content compared with the DMN-treated group (Fig. 4C). These data suggest that AF can reduce DMN-induced expression of α-SMA and collagen types I and III through activated HSCs.

3.4. AF reduces DMN-induced expression of PDGFR-β, TGF-β1 and TNF-α

Various cytokines and cellular effectors play an important role in HSCs turnover in the fibrotic process (Fallowfield and Iredale, 2004; Lin et al., 2008). We evaluated the effects of AF on the levels of TGF-β1, TNF-α and PDGFR-β expression in liver tissue as determined by quantitative RT-PCR and western blotting analysis. As shown in Fig. 5, the expression of TGF-β1, TNF-α and PDGFR-β were increased in DMN-treated group compared with control group, consistent with DMN-induced hepatotoxicity. Levels of TGF-β1, TNF-α and PDGFR-β decreased compared with those of the DMN-treated group when AF was administrated (Fig. 5A and B).

4. Discussion

Hepatic fibrosis involves the formation or development of excess fibrous connective tissue as a result of liver injury. Chronic liver disease can progress to cirrhosis of the liver and end-stage liver disease, which manifests as portal hypertension, synthetic dysfunction, hepatopulmonary syndrome, and encephalopathy and hepatocellular carcinoma. Cirrhosis of the liver cause death, human suffering, and hospital costs. Better therapies for combating hepatic fibrosis and cirrhosis are thus needed. The inhibition and prevention of the development of fibrosis might be an effective strategy to improve the prognosis of patients with chronic liver

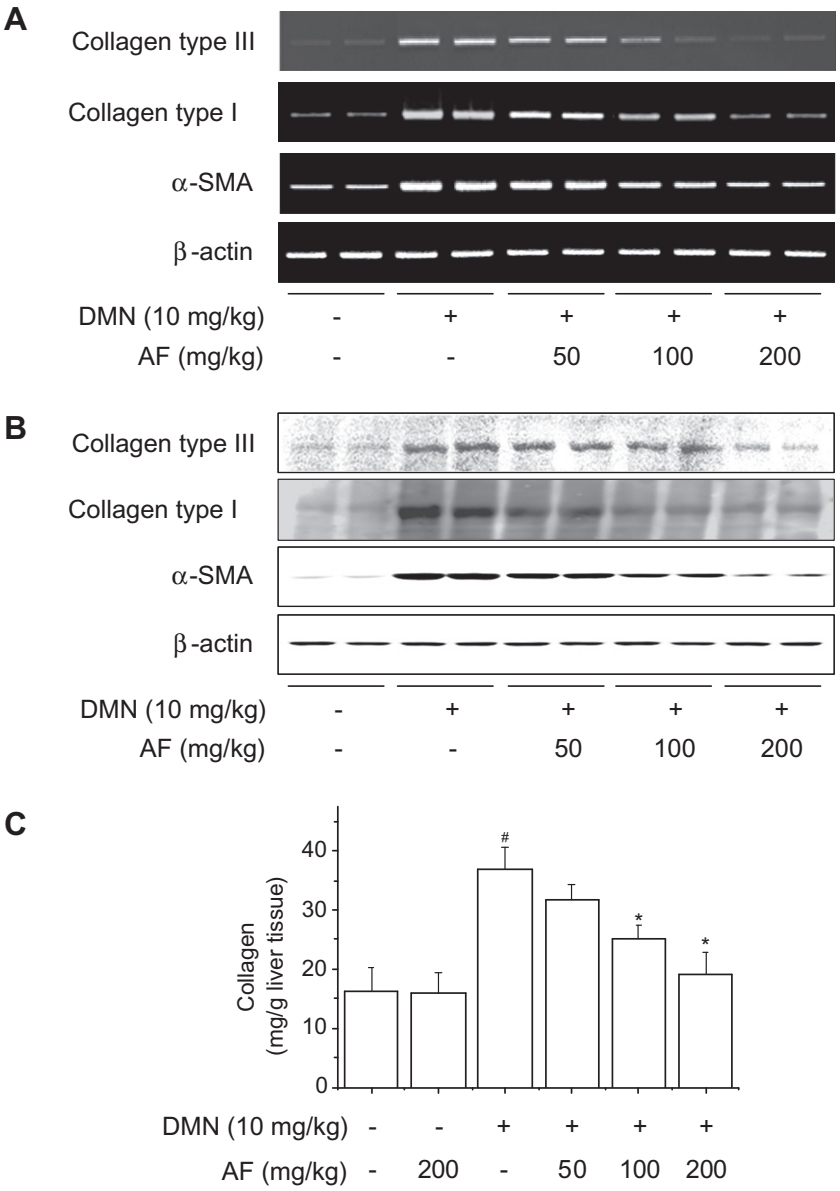

Fig. 4. Effects of AF on the DMN-induced expression of α-SMA and collagen types I and III. (A) RT-PCR and (B) western blotting analysis of α-SMA and collagen type I and III mRNA and protein expression were performed on the livers of fibrotic rats. (C) Liver collagen content was determined by assaying total soluble collagen using the Sircol collagen assay kit according to the manufacturer's directions. Results are the mean ± SEM for five rats in each group. Results were obtained from three independent experiments. ($^{\#}P < 0.05$) significantly different from the control group. ($^{*}P < 0.05$) significantly different from the DMN-treated group.

injury. Recent these agents make the use of natural agents. Attention is now being focused on purple sweet potato because of its unique color, nutrition and health-promoting benefits (Lila, 2004). Here, we extended these investigations by assessing the anti-fibrotic effects of anthocyanin fraction (AF) obtained from the purple-fleshed sweet potato on hepatic fibrosis induced by DMN in Sprague–Dawley rats.

Experimental animal models of hepatic fibrosis use DMN administration to simulate the clinical features of human liver fibrosis such as mortality, ascites, hepatic parenchymal cell destruction, formation of connective tissue, and nodular regeneration (George et al., 2001; Bataller and Brenner, 2005). This system provides a preclinical model for evaluating the therapeutic efficacy of a drug and the underlying mechanisms of its mode of action (Kang et al., 2002).

Anthocyanins represent a group of water-soluble phenolic compounds responsible for the blue, red, and purple colors of many fruits and vegetables. Experimental studies have shown that anthocyanins protect mouse liver from D-galactose-induced injury by attenuating oxidative stress and suppressing inflammation and histological changes (Zhang et al., 2009). In addition, anthocyanins have been recommended as chemo-preventative phytochemicals, as they can stimulate the antioxidant system to resist oxidant-induced injury (Shih et al., 2007).

In current studies demonstrate that AF can significantly reduce DMN-induced serum ALT and AST activity (Fig. 2A and B) and can lead to a recovery of reduced body and liver weight (Fig. 2C and D) after 4 weeks of treatment. In addition, histological examination showed that AF decreases DMN-induced liver fibrosis (Fig. 3A and B) and fibrosis scores (Fig. 3C). Liver fibrosis is not only the result of derangements in the synthesis and degradation of matrix, but also the result of liver injury. Thus, these data implicates that the improvement of DMN-induced hepatic fibrosis by AF may result partially from attenuation of hepatocytes injury.

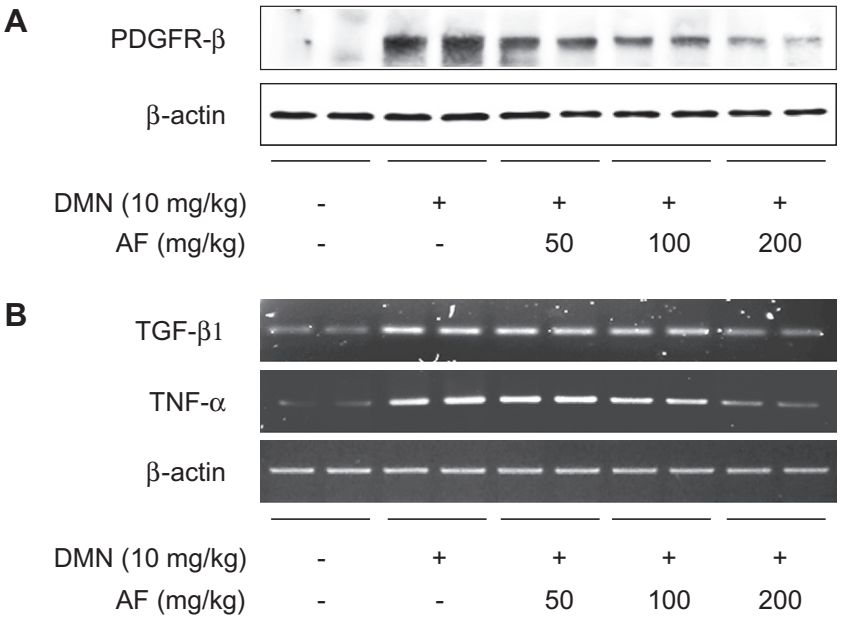

Fig. 5. Effects of AF on the DMN-induced expression of PDGFR-β, TGF-β1 and TNF-α. (A) PDGFR-β protein and (B) TGF-β1 and TNF-α mRNA expression were performed on the livers of fibrotic rats. Results were obtained from three independent experiments.

Activated HSCs transition into myofibroblast-like cells that express α-SMA, and these activated HSCs secrete ECM proteins during hepatic fibrosis (Pinzani et al., 1998). Furthermore, α-SMA is a marker for the early stage of hepatic fibrosis (Carpino et al., 2005). Our present data show that DMN increased the number of α-SMA positive cells in the liver and these proliferations are suppressed by AF ingestion. Taken together, these findings suggest the anti-fibrotic effect of AF may be due to, at least, by the suppression of HSC activation.

Hepatic fibrosis is chronic inflammatory response to injury. The excessive ROS causing the depletion of endogenous antioxidants directly or indirectly contribute to the accumulation of ECM via stimulation of HSCs and induction of fibrogenic cytokines, such as TGF-β, PDGFR-β, and TNF-α (Bataller and Brenner, 2005). Our current studies demonstrate that AF reduces markers of fibrosis process, such as inhibitions in TGF-β1, TNF-α and PDGFR-β expression in the liver (Fig. 5A and B). This finding regarding the gene level correlated with the protein levels of PDGFR-β and TGF-β1 in liver tissue. PDGFR-β and TGF-β1 originate primarily from activated Kupffer cells, apoptotic hepatocytes, and activated HSCs, and then synergistically causes the transformation of HSCs into myofibroblasts secreting ECM (Friedman, 2008). Thus, the anti-fibrotic effect of AF is partially due to inhibition of HSC proliferation through down-regulation of TGF-β1, TNF-α and PDGFR-β gene.

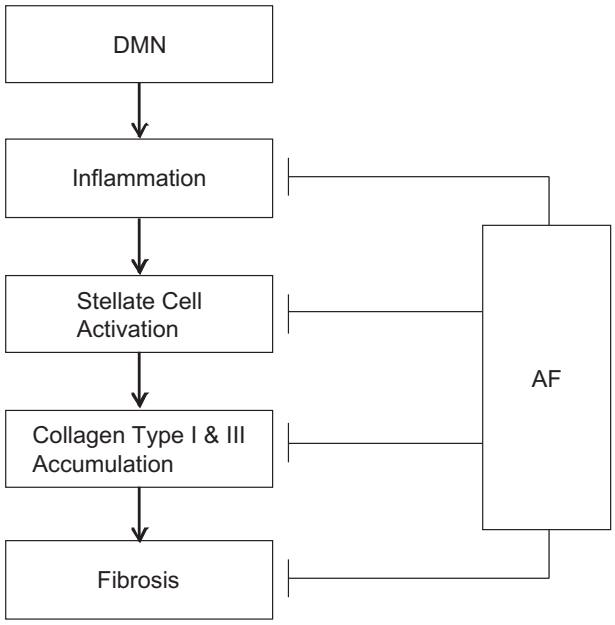

Fig. 6. Diagram shows that protective mechanisms of AF against DMN-induced hepatic fibrosis in rats. AF can act via different mechanisms to reduce HSCs activation and prevent collagen accumulations. These different mechanisms including antioxidant, hepatoprotective and anti-inflammatory effects.

In summary, the present study demonstrated that AF exhibited *in vivo* hepatoprotective and anti-fibrotic effects against liver injuries induced by DMN. The mechanism appeared mostly to be mediated by inactivation of HSCs but also could involve inhibition of the α-SMA, hepatic collagen, PDGFR-β, TGF-β1, and TNF-α production (Fig. 6). In addition, AF might also possess beneficial effect on the restoration of impaired oxidative balance as an efficient antioxidant, which exerts a protective effect against HSC activation. Our data suggests that AF may be potentially useful in the prevention of the development of hepatic fibrosis.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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