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The Proanthocyanidins Inhibit Dimethylnitrosamine-induced Liver Damage in Rats

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Proanthocyanidins are naturally occurring compounds widely available in fruits, vegetables, nuts and seeds. They are a class of phenolic compounds and have been reported to exhibit a wide range of biological effects. In this study, we investigated the protective effect of grape seed proanthocyanidins on hepatic injury induced by dimethylnitrosamine (DMN) in rats. Treatment with DMN caused a significant increase in levels of serum alanine transaminase, aspartate transaminase, alkaline phosphatase, and bilirubin. Oral administration of proanthocyanidins (20 mg/kg daily for 4 weeks) remarkably prevented these elevations. Proanthocyanidins also restored serum albumin and total protein levels, and reduced the hepatic level of malondialdehyde. Furthermore, DMN-induced collagen accumulation, as estimated by histological analysis of liver tissue stained with Sirius red, was reduced in the proanthocyanidins-treated rats. A reduction in hepatic stellate cell activation, as assessed by α-smooth muscle actin staining, was associated with proanthocyanidins treatment. In conclusion, these results demonstrate that proanthocyanidins exhibited *in vivo* hepatoprotective and anti-fibrogenic effects against DMN-induced liver injury. It suggests that grape seed proanthocyanidins may be useful in preventing the development of hepatic fibrosis.

Key words: Proanthocyanidins, Grape seed, Hepatoprotective effect, Anti-fibrogenic effect, Liver fibrosis, Hepatic stellate cell

INTRODUCTION

Hepatic fibrosis is a wound-healing process in livers with chronic injury and is characterized by the excess production and deposition of extracellular matrix (ECM) components. Viral infection, alcoholic or drug toxicity, or any other factors that cause damage to hepatocytes elicit an inflammatory reaction in the liver. The damaged hepatocytes, their membrane components, metabolites of toxic agents, and infiltrating inflammatory cells are the activators of Kupffer cells. The activated Kupffer cells release a number of soluble agents, including cytokines, reactive oxygen species (ROS), and other factors (Kolios et al., 2006; McMullen et al., 2008). These factors act on the hepatic stellate cells (HSCs), those are localized in the para-sinusoidal space and store most of the vitamin A in the body. HSCs are normally quiescent and produce small amounts of ECM components, such as laminin and collagen type IV for the formation of basement membranes (Maher and Bissell, 1993; Moreira, 2007). When exposed to soluble factors from damaged hepatocytes or activated Kupffer cells, HSCs lose their lipid content, undergo morphological transition to myofibroblast-like cells. This transition is characterized by an accelerated production of large amounts of ECM (Friedman, 2008a). During this complicated cross-talking of various cell types, mediated by different cytokines and other soluble factors, hepatocellular damage is an initiating event, activated Kupffer cells serve as the mediator, and HSCs act as the effectors (Pinzani et al., 1998). Therefore, all these cell types are targets for pharmacological or molecular interven-

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tions for the treatment of hepatic fibrosis.

Proanthocyanidins, also known as condensed tannins, are polymeric compounds, the basic structural elements of which are polyhydroxyflavan-3-ol units linked together by carbon-carbon bonds. They are widely distributed in the plant kingdom and represent one of the most abundant groups of higher plant secondary metabolites (Gabetta et al., 2000). Proanthocyanidins have been reported to possess a broad spectrum of pharmacological and medicinal properties against oxidative stress (Bagchi et al., 2002). Besides the free radical scavenging and antioxidant activity, proanthocyanidins exhibit vasodilatory, anticarcinogenic, antiallergic, anti-inflammatory, antibacterial, cardioprotective, immune-stimulating, anti-viral and estrogenic activities. Furthermore, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility. They also modulate the activity of enzyme systems including phospholipase A_2, cyclooxygenase and lipoxygenase (Bagchi et al., 2000). Recently grape seed extract containing a number of polyphenol such as procyanidins and proanthocyanidins was reported to reduce oxidative stress and fibrosis in experimental biliary obstruction in rats (Dulundu et al., 2007).

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen (Haggerty and Holsapple, 1990). At doses as small as 20 mg/kg, DMN can cause massive liver necrosis and death in many species (Nakamura et al., 2004). Exposure to repeated lower doses of DMN causes subacute and chronic liver injury with varying degrees of necrosis, fibrosis, and nodular regeneration (Terracini et al., 1967). DMN-induced fibrosis model is known to reproduce most of the features observed during human liver fibrosis (Weng et al., 2001). This model has several benefits, such as progressive and remarkable pathological alteration, high reproduction rate of fibrosis and low mortality rate in experimental animals (Jezequel et al., 1989).

The present study was designed to determine whether proanthocyanidins have an antifibrogenic effect on DMN-induced hepatic fibrosis in rats.

MATERIALS AND METHODS

Chemicals

A commercially available dried, powered Graseedex[TM] (batch no. 100# 20020814) was obtained from MSC Ind. Co., LTD. The content of proanthocyanidins was more than 97%, which was determined by the vanillin-HCl method of Broadhust and Jones (1978) using (+)-cathechin as a standard.

Induction of liver fibrosis with DMN

Animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. Male Sprague-Dawley (SD) rats were obtained from Dae-Han Laboratory Animal Research Center Co., Ltd. Animals were kept on standard rat chow with free access to tap water, in a temperature- and humidity-controlled animal house under 12-h light-dark cycles. Eighteen rats weighing 140-160 g were divided into 3 groups of 6 each: Normal, DMN and Pro20 groups. The Pro20 group was treated with intraperitoneal injections of DMN (diluted with saline) at a dose of 10 mg/kg body weight per day for 3 consecutive days per week for 4 weeks (Jezequel et al., 1989; Lee et al., 2003), and treated daily with proanthocyanidins (suspended in 0.5% carboxymethylcellulose sodium, CMC) at a dose of 20 mg/kg by oral gavage for the length of the study, respectively. The DMN group was treated with DMN as described above and equivalent volumes of 0.5% CMC solution. The normal group was treated with the volume of saline and 0.5% CMC solution equivalent to those of the proanthocyanidins group. At the end of the fourth week, all rats were sacrificed under ether anesthesia and their livers were excised and weighed. Blood samples for biochemical analyses were obtained from the inferior *vena cava*. The liver specimens were immediately fixed in 10% neutral buffered formalin (NBF) for histochemical studies. The remaining liver tissue was homogenized using glass Potter-Elvehjem homogenizer set. The homogenate was freed from the cellular debris and nuclei by centrifugation at $700 \times g$ at 4°C for 10 min. Protein concentration were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Biochemical analyses of serum

Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) activities were estimated by colorimetric methods using commercial kits (Eiken). Serum albumin, total protein and bilirubin levels were also measured using commercial kits following the manufacturer's protocols.

Histology and immunohistochemistry

Five-micrometer liver sections were deparaffinized and processed routinely for hematoxylin-eosin (H&E) and Sirius red (SR) staining (Junqueira et al., 1979). And they were examined immunohistochemically for α-smooth muscle actin (α-SMA, Serotec) using routine indirect avidin-biotin immunolabelling procedures. Non-immune isotype-matched immunoglobulin was used in

place of the primary antibody in the negative controls.

Image analysis

The mean values of SR or α-SMA positive areas were assessed in 6 ocular fields per specimen, which were randomly selected at 40 x magnification using an image analysis system (Image Pro Plus 4.0, Media Cybernetics). The SR or α-SMA positive areas were determined as the mean of triplicate examinations and expressed as a percentage of the total area of the specimen.

Determination of malondialdehyde contents

MDA levels in liver homogenates of the normal, the DMN and the proanthocyanidins treated rats were determined by the Buege and Aust method (Buege and Aust, 1978).

Antioxidative effect of proanthocyanidins

Lipid peroxidation in rat liver homogenate induced by Fenton reaction composing of 0.1 mM $FeSO_4$, 3 mM H_2O_2, various concentrations of the tested substances and liver homogenate (7.5 mg protein/mL) were measured by the method of Buege and Aust (Buege and Aust, 1978) with some modification (Moon et al., 1998). The reaction was started by the addition of $FeSO_4$ and H_2O_2 and then incubated at 37°C for 10 min. The reaction was stopped by mixing with 3 mL of a stock solution of 15% (w/v) trichloroacetic acid, 0.375 % (w/v) thiobarbituric acid, 0.125 M hydrochloric acid, and 0.6 mM butylhydroxytoluene (BHT). The combination of reaction mixture and stock solution was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at $1250 \times g$ for 20 min. The absorbance of the supernatant was determined at 532 nm and the MDA concentration was calculated using 1,1,3,3-tetraethoxypropane as a standard.

Statistical analyses

All values were expressed as the mean ± S.E. Significant differences between the normal, DMN and DMN plus proanthocyanidins were statistically analyzed using a one-way analysis of variance (ANOVA), followed by a non-parametric post hoc test (LSD). A p value for 0.05 or less was considered statistically significant.

RESULTS

Serum parameters of liver function

The effects of proanthocyanidins on serum parameters in the liver fibrosis model are shown in Table I. DMN-induced increase in serum AST, ALT, ALP and bilirubin levels were significantly suppressed by proanthocyanidins treatment. In the case of chronic liver diseases, the serum albumin level is reduced due to protein synthesis disorder in the liver. In the Pro20 group, diminished serum albumin and total protein concentrations were increased. The liver occupies a central role in the metabolism of bile pigments in the phase of hepatic uptake, conjugation and excretion phases. Excretion of bile pigments is susceptible to impairment when the liver cell is damaged. Proanthocyanidins inhibited the increases in the plasma bilirubin content in rats treated with DMN. These results indicate that proanthocyanidins protected the necrosis of hepatocytes due to DMN administration.

Histopathology and immunohistochemistry

The effects of proanthocyanidins on DMN-induced liver injury were evaluated by histopathologic examination of the liver sections by H&E staining. In contrast to the normal group of rats (Fig. 1A1), the

Table I. Effects of proanthocyanidins on serum parameters with respect to liver functions of rats treated with DMN for 4 weeks

	Normal	DMN	Pro20
AST (U/L)	21.6 ± 2.5	150.4 ± 24.8$^{###}$	33.3 ± 3.8***
ALT (U/L)	18.7 ± 2.6	95.7 ± 11.7$^{###}$	42.2 ± 5.3***
ALP (U/L)	16.0 ± 0.8	136.1 ± 16.0$^{###}$	51.5 ± 6.5***
Albumin (g/dl)	2.54 ± 0.06	2.25 ± 0.05$^{##}$	2.47 ± 0.07*
T. Protein (g/dl)	5.75 ± 0.17	4.77 ± 0.36$^{##}$	5.64 ± 0.09*
D. Bilirubin (mg/dl)	0.03 ± 0.02	0.22 ± 0.02$^{##}$	0.08 ± 0.05*
T. Bilirubin (mg/dl)	0.15 ± 0.04	0.41 ± 0.05$^{###}$	0.18 ± 0.02***

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except normal group. Values are the mean ± S.E. of 6 rats. Statistical significance: $^{*}p < 0.05$, $^{***}p < 0.001$ vs. DMN, and $^{##}p < 0.01$, $^{###}p < 0.001$ vs. normal, respectively. DMN, DMN alone; Pro20, DMN with 20 mg /kg/day proanthocyanidins by oral gavage. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T. Protein, total protein ; D. Bilirubin, Direct bilirubin; T. Bilirubin, total bilirubin.

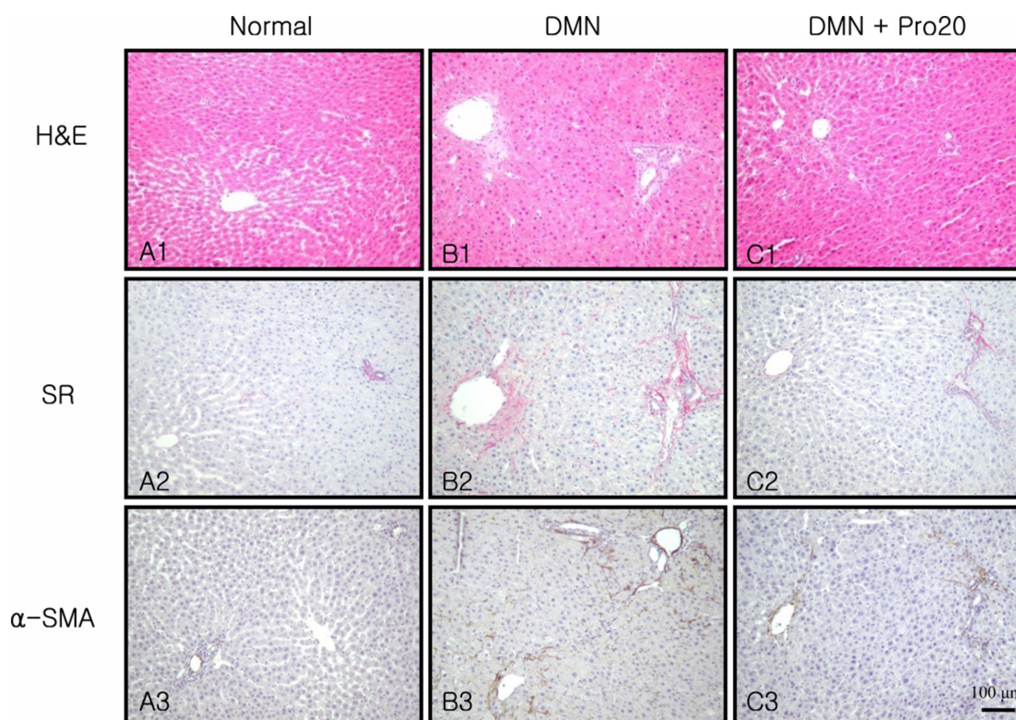

Fig. 1. Histological analysis of liver sections. (**A**) Normal. (**B**) DMN (10 mg/kg per day for consecutive days of each week for 4 weeks) alone-treated group. (**C**) DMN with proanthocyanidins (20 mg/kg/d *p.o.*)-treated group. The sections were stained with hematoxylin-eosin (H&E) and with Sirius red (SR). Activated HSCs were detected by immunohistochemistry with α-SMA antibody (α-SMA).

dispensation of DMN for 4 weeks caused extensive hemorrhagic necrosis and disruption of tissue architecture (Fig. 1B2). These alterations were remarkably reduced in the liver sections of the proanthocyanidins-treated rats (Fig. 1C1). Serial sections were stained with Sirius Red for collagen. In the liver sections taken from the normal, collagen fibers were observed only in the periportal area (Fig. 1A2). The livers of the DMN-treated rats exhibited an increase in collagen content, and displayed bundles of collagen fibers surrounding the lobules, forming large fibrous septa (Fig. 1B2). The thickening of these collagen fiber bundles was markedly reduced in the proanthocyanidins group (Fig. 1C2). Quantitative analysis by an image analysis technique also showed a remarkable reduction of SR staining regions in the proanthocyanidins treated groups compared with the DMN group (Table II).

The expression of α-SMA, an indicator of activated HSCs was detected by the immunohistochemistry method (Friedman, 2000). In contrast to the normal group (Fig. 1A3), many α-SMA-positive cells were detected around the periportal fibrotic band areas and were scattered in the regions of connective tissue septa in the DMN-treated rats (Fig. 1B3). However, in proanthocyanidins treated group, only traces of α-SMA-positive cells were detected. The level of α-SMA expression was almost the same as in the control liver of the control (Fig. 1C3). Quantitative analysis by an image analysis technique showed a remarkable reduction of α-SMA-positive regions in the proanthocyanidins treated group compared with the DMN group (Table II).

Table II. Effect of proanthocyanidins treatment on the Sirius red-staining or α-SMA-immunoreactive regions

Treatment	Sirius red-staining regions (%)	α-SMA-positive regions (%)
Normal	0.10 ± 0.02	0.05 ± 0.01
DMN	$4.81 \pm 1.12^{\#\#\#}$	$2.33 \pm 0.35^{\#\#\#}$
Pro20	$0.41 \pm 0.08^{***}$	$0.09 \pm 0.05^{***}$

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except normal group. Values are the mean ± S.E. of 6 rats. Statistical significance: $^{***}p < 0.001$ vs. DMN, and $^{\#\#\#}p < 0.001$ vs. normal, respectively. DMN, DMN alone; Pro20, DMN with 20 mg/kg/day proanthocyanidins by oral gavage.

Lipid peroxidation

The lipid peroxidation in the liver was measured by MDA determination in liver homogenate. As shown in

Table III. Effect of proanthocyanidins on the malondialdehyde (MDA) contents in the liver of rats treated with DMN

Treatment	Normal	DMN	Pro20
MDA (nmole/mg protein)	0.20 ± 0.01	0.34 ± 0.02$^{###}$	0.27 ± 0.01**

Data represent the means ± S.E. of six rats. $^{###}p < 0.001$ when compared to the normal group. $^{**}p < 0.01$, when compared to the DMN group.

Table IV. Effects of proanthocyanidins on the lipid peroxidation of rat liver homogenate induced by $FeSO_4$ and H_2O_2

Substance	Concentration (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Butylhydroxytoluene	0.17	19.6 ± 0.8	4.2
	1.73	47.5 ± 0.9	
	17.30	62.3 ± 0.1	
Proanthocyanidins	0.32	10.4 ± 1.1	15.5
	3.20	29.7 ± 0.3	
	32.00	53.1 ± 0.4	

Results are expressed as mean ± S.E. (n=3). The reaction mixture (1.0 mL) was composed of the rat liver homogenate (7.5 mg prot.), 0.1 mM $FeSO_4$, 3 mM H_2O_2, and various concentration of buthyhydroxytoluene or proanthocyanidins. After incubation at 37°C for 10 min, the amount of malondialdehyde (MDA) formation was measured by method of Buege and Aust (1978). Inhibition (%) of MDA formation of butylhydroxytoluene or proanthocyanidins was calculated based on the amount of MDA formation of the $FeSO_4/H_2O_2$ treated control (24.4 μM) after subtracting the normal (6.8 μM).

Table III, the MDA content in the DMN group was higher than that of the normal group (165% of normal) and in the Pro20 group, it provided 52% of protection. These data indicate that proanthocyanidins treatment inhibited lipid peroxidation in the DMN-induced liver damage.

Antioxidative activity of proanthocyanidins

Antioxidant activity of proanthocyanidins was estimated by examination of the inhibitory effect against $FeSO_4/H_2O_2$-inducd lipid peroxidation on rat liver homogenate and results are shown in Table IV. As a positive control, BHT was also tested. BHT is a well-known chain-breaking antioxidant and it had high inhibitory effect against $FeSO_4/H_2O_2$-inducd lipid peroxidation with IC_{50} values of 4.2 μg/mL. IC_{50} value of proanthocyanidins was 15.5 μg/mL.

DISCUSSION

Hepatic fibrosis represents a common response to chronic liver injuries of variable origin, *e.g.*, viral, metabolic and toxic. Regardless of the type of insults, liver fibrosis is characterized by the increased production of extracellular matrix (ECM) proteins. Hepatic fibro-genesis is accompanied by hepatocellular necrosis and inflammation. HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver (Moreira, 2007), and activated HSCs have been identified as the primary source of excess accumulation of ECM components in liver fibrosis.

In this study, proanthocyanidins protected the hepatocytes from injuries and improved the liver function of the DMN-treated rats. The damaged hepatocytes are potent sources of reactive oxygen intermediates and these compounds exert paracrine stimulation of stellate cells. Therefore, the hepatoprotective effects of proanthocyanidins may decrease paracrine stimuli, which lead to hepatic fibrosis via activated HSCs. Our present data show that DMN increased the number of α-SMA positive cells in the liver and that these proliferations were suppressed by proanthocyanidins ingestion and that proanthocyanidins significantly suppressed the increased collagen accumulated in the DMN-induced liver injury in rats. Taken together, these findings suggest that the antifibrotic effect of proanthocyanidins may be due to, at least, suppressed HSC activation via hepatoprotective effect.

Oxidative stress plays an important role in many types of acute liver injury (Zhu and Fung, 2000; Dryden et al., 2005; Medina and Moreno-Otero, 2005). Much experimental and clinical data indicate that a common link between chronic liver damage and hepatic fibrosis may be related to oxidative stress (Vendemiale et al., 2001; Bruck et al., 2007; Karaa et al., 2008), which has been reported to be associated with the HSC activation (Friedman, 2008b). There is sufficient evidence suggesting that lipid peroxidation can occur in both acute and chronic liver injuries (Chen et al., 2001). It has been shown that certain lipid peroxidation products induce genetic over-expression of fibrogenic cytokines and increase the synthesis of collagen by initiating the activation of HSCs (Parola et al., 1993; Nieto and Cederbaum, 2005). Therefore, reducing oxidative stress, which is an important stimulus to activation of HSCs, is a relatively practical avenue of intervention (Albanis and Friedman, 2006). This study showed that the DMN-treated rats exhibited increased levels of hepatic MDA and that proanthocyanidins reduced this increase. These outcomes suggest that the mechanism for the hepatoprotective effects of proanthocyanidins in the development of liver fibrosis may be related to the reduction of lipid peroxidation by its antioxidative activity.

In summary, the present study demonstrated that proanthocyanidins exhibited *in vivo* hepatoprotective and antifibrotic effects against liver injury induced by DMN. The mechanism appeared mostly to be mediated by inactivation of HSCs. In addition, proanthocyanidins might also produce beneficial effects by reducing oxidative stress in DMN-treated rats, which exerted protective effects against HSC activation. Our data suggest that proanthocyanidins, one of the most abundant polyphenols in grape seeds may be potentially useful in the prevention of the development of hepatic fibrosis.

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