The Flavonoid Naringenin Inhibits Dimethylnitrosamine-Induced Liver Damage in Rats

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Naringenin, a phytoalexin found in grapefruits and tomatoes, has been reported to exhibit a wide range of pharmacological properties. In this study, we investigated the protective effect of naringenin on hepatic injury induced by dimethylnitrosamine (DMN) in rats. Oral administration of naringenin (20 and 50 mg/kg daily for 4 weeks) remarkably prevented the DMN-induced loss in body and liver weights and inhibited the elevation of serum alanine transaminase, aspartate transaminase, alkaline phosphatase, and bilirubin levels. Naringenin 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 naringenin-treated rats. A reduction in hepatic stellate cell activation, as assessed by α -smooth muscle actin staining, was associated with naringenin treatment. In conclusion, these results demonstrate that naringenin exhibited *in vivo* hepatoprotective and anti-fibrogenic effects against DMN-induced liver injury. It suggests that naringenin may be useful in preventing the development of hepatic fibrosis.

Key words naringenin; hepatoprotective effect; anti-fibrogenic effect

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.¹⁾ 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.²⁾ When exposed to soluble factors from damaged hepatocytes or activated Kupffer cells, HSCs lose their lipid content, undergo morphological transition to myofibroblast-like cells and proliferate. This transition is characterized by an accelerated production of large amounts of ECM.^{3,4)} 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.⁵⁾ Therefore, all these cell types are targets for pharmacological or molecular interventions for the treatment of hepatic fibrosis.

Naringenin (4',5,7-trihydroxyflavanone) (Fig. 1), aglycone of naringin, is a predominant flavanone in grapefruits and tomatoes. It has been reported to have several biological effects, such as an anticancer,^{6,7)} antimutagenic,^{8,9)} anti-inflammatory^{10,11)} and anti-atherogenic activity.¹²⁾ Recently, naringenin was found to protect cultured rat hepatocytes from *tert*-butylhydroperoxide (*t*-BHP) toxicity,¹³⁾ alter antioxidant enzyme activities of erythrocytes and liver in high cholesterol-fed rats, and reduce their oxidative stress intensity.¹⁴⁾ Although the wide range of biological effects exhibited by naringenin has been reported, no information is

available on its antifibrotic efficacy.

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen.¹⁵⁾ At doses as small as 20 mg/kg, DMN can cause massive liver necrosis and death in many species.¹⁶⁾ Exposure to repeated lower doses of DMN causes subacute and chronic liver injury with varying degrees of necrosis, fibrosis, and nodular regeneration.¹⁷⁾ DMN-induced fibrosis model is known to reproduce most of the features observed during human liver fibrosis.¹⁸⁾ This model has benefits, such as progressive and remarkable pathological alteration, a high reproduction rate of fibrosis and a low mortality rate in experimental animals.¹⁹⁾ An experimental liver fibrosis model induced by DMN in rats has been used in this study.

In view of the protective effects of naringenin against a variety of toxicants and carcinogens, the present study was designed to determine whether naringenin has an antifibrogenic effect on DMN-induced hepatic fibrosis in rats.

MATERIALS AND METHODS

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 rats were obtained from Dae-Han Laboratory Animal Research Center Co., Ltd. (Seoul, Korea). 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. Twenty-four rats, 140–160 g were divided

Fig. 1. Chemical Structure of Naringenin (4',5,7-Trihydroxyflavanone)

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into 4 groups of 6 each: control, DMN, Nar20 and Nar50 group. The Nar20 or Nar50 group was treated with intraperitoneal injections of DMN (diluted with saline) at a dose of 10 mg/kg of body weight per day for 3 consecutive days per week for 4 weeks,²⁰⁾ and treated daily with naringenin (suspended in 0.5% carboxymethylcellulose sodium, CMC) at a dose of 20 or 50 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 control group was treated with the volume of saline and 0.5% CMC solution equivalent to those of the naringenin groups. 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 for histochemical studies. The remaining liver tissue was homogenized using a 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 concentrations were determined by the method of Lowry *et al.* using bovine serum albumin as the standard.²¹⁾

Biochemical Analyses of Serum Serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activities were estimated by colorimetric methods using commercial kits (Eiken, Tokyo, Japan). 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,²²⁾ and were examined immunohistochemically for α -smooth muscle actin (α -SMA, Serotec, Oxford, U.K.) using routine indirect avidin-biotin immunolabeling 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 $40\times$ magnification using an image analysis system (Image Pro Plus 4.0, Media Cybernetics, Del Mar, CA, U.S.A.). 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 (MDA) Contents MDA levels in liver homogenates of the control, the DMN and the naringenin treated rats were determined by the Buege and Aust method.²³⁾

Antioxidative Effect of Naringenin 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) was measured by the method of Buege and Aust²³⁾ with some modification.²⁴⁾ 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 the 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 $\pm$ S.E. Significant differences between the groups were statistically analyzed using a one-way analysis of variance (ANOVA), followed by a non-parametric post hoc test (LSD). A *p* value of 0.05 or less was considered statistically significant.

RESULTS

Body and Liver Weights The effects of naringenin on body weight and liver weight of rats are shown in Fig. 2 and Table 1. Treatment with DMN caused a significant decrease in both body weight and liver weight compared with the control group. In the DMN group, body weight gain for 4 weeks was only 38% of the control and oral administration of naringenin decreased this loss. In the Nar20 and Nar50 group, the body weight gains were 65% and 71% of the control, respectively. In the DMN group, the liver weight was 69% of the control. But naringenin administration significantly prevented this DMN-induced loss of liver weight: liver weights in the Nar20 and Nar50 group were 78% and 85% of the control, respectively.

Serum Parameters of Liver Function The effects of naringenin on serum parameters in the liver fibrosis model are shown in Table 2. DMN-induced increases in serum AST, ALT, ALP and bilirubin levels were significantly suppressed

Fig. 2. Effect of Naringenin on the Change of Body Weight

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except control group. DMN, DMN alone; Nar20, DMN with 20 mg/kg/d naringenin by oral gavage; Nar50, DMN with 50 mg/kg/d naringenin by oral gavage.

Table 1. Effects of Naringenin on Body and Liver Weights of Rats Treated with DMN

	Body weight (g)	Liver weight (g)
Control	345 $\pm$ 10	11.4 $\pm$ 0.6
DMN	238 $\pm$ 19 ^{###}	7.1 $\pm$ 0.9 ^{###}
Nar20	285 $\pm$ 6*	9.0 $\pm$ 0.2*
Nar50	295 $\pm$ 7**	9.7 $\pm$ 0.4**

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except the control group. Values are mean $\pm$ S.E. of 6 rats. Statistical significance: **p* < 0.05, ***p* < 0.01 vs. DMN, and ^{###}*p* < 0.001 vs. control, respectively. DMN, DMN alone; Nar20, DMN with 20 mg/kg/d naringenin by oral gavage; Nar50, DMN with 50 mg/kg/d naringenin by oral gavage.

Table 2. Effects of Naringenin on Serum Parameters with Respect to Liver Functions of Rats Treated with DMN for 4 Weeks

	Control	DMN	Nar20	Nar50
AST (U/l)	27.4±3.4	126.6±23.3 ^{###}	68.8±4.0 ^{**}	42.1±2.9 ^{***}
ALT (U/l)	14.8±3.1	83.9±16.3 ^{###}	33.3±1.1 ^{***}	43.6±2.5 ^{**}
ALP (U/l)	15.9±0.8	138.2±16.0 ^{###}	49.1±4.9 ^{***}	43.1±1.9 ^{***}
Albumin (g/dl)	1.99±0.09	1.53±0.17 ^{##}	1.92±0.06 [*]	1.95±0.05 [*]
T. Protein (g/dl)	5.53±0.15	4.69±0.46 ^{##}	5.69±0.09 ^{**}	5.61±0.10 ^{**}
D. Bilirubin (mg/dl)	0.04±0.02	0.22±0.04 ^{###}	0.09±0.01 ^{**}	0.06±0.02 ^{***}
T. Bilirubin (mg/dl)	0.15±0.04	0.36±0.05 ^{##}	0.24±0.02 [*]	0.19±0.01 ^{**}

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except control group. Values are mean±S.E. of 6 rats. Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. DMN, and # $p<0.01$, ### $p<0.001$ vs. control, respectively. DMN, DMN alone; Nar20, DMN with 20 mg/kg/d naringenin by oral gavage; Nar50, DMN with 50 mg/kg/d naringenin 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. 3. Histological Analysis of Liver Sections

(A) Control. (B) DMN (10 mg/kg per day for 3 consecutive days of each week for 4 weeks) alone-treated group. (C) DMN with naringenin (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). Note the increase in the α -SMA-positive stellate cells that synthesize collagen after DMN treatment compared with the control. When treated with naringenin very few activated HSCs were observed and they synthesized traces of collagen.

by naringenin treatment. In the case of chronic liver diseases, the serum albumin level is reduced due to protein synthesis disorder in the liver. Again, in the Nar20 and Nar50 groups, diminished serum albumin and total protein concentrations were restored to the control levels. The liver occupies a central role in the metabolism of bile pigments in the hepatic uptake, conjugation and excretion phases. Excretion of bile pigments is susceptible to impairment when the liver cell is damaged. Naringenin inhibited the increases in the plasma bilirubin content in rats treated with DMN. These results indicate that naringenin protected the necrosis of hepatocytes due to DMN administration.

Histopathology and Immunohistochemistry The effects of naringenin on DMN-induced liver injury were evaluated by histopathologic examination of the liver sections by H&E staining. In contrast to the control group of rats (Fig. 3A1), the dispensation of DMN for 4 weeks caused extensive hemorrhagic necrosis and disruption of tissue architecture

(Fig. 3A2). These alterations were remarkably reduced in the liver sections of the naringenin-treated rats (Fig. 3A3). Serial sections were stained with SR for collagen. In the liver sections taken from the control, collagen fibers were observed only in the periportal area (Fig. 3B1). 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. 3B2). The thickening of these collagen fiber bundles was markedly reduced in the naringenin groups (Fig. 3B3). Quantitative analysis by an image analysis technique also showed a remarkable reduction of SR staining regions in the naringenin treated groups compared with the DMN group (Table 3).

The expression of α -SMA, an indicator of activated HSCs, was detected by the immunohistochemistry method.^{25,26} In contrast to the control group (Fig. 3C1), many α -SMA-positive cells were detected around the periportal fibrotic band areas and were scattered in the regions of connective tissue

Table 3. Effect of Naringenin Treatment on the Sirius Red-Staining or α -SMA-Immunoreactive Regions

Treatment	Sirius red-staining regions (%)	α -SMA-positive regions (%)
Control	0.14 $\pm$ 0.05	0.02 $\pm$ 0.01
DMN	2.33 $\pm$ 0.35 ^{###}	3.05 $\pm$ 0.39 ^{###}
Nar20	0.28 $\pm$ 0.06 ^{***}	0.07 $\pm$ 0.01 ^{***}
Nar50	0.06 $\pm$ 0.00 ^{***}	0.07 $\pm$ 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 the control group. Values are mean $\pm$ S.E. of 6 rats. Statistical significance: *** p <0.001 vs. DMN, and ^{###} p <0.001 vs. control, respectively. DMN, DMN alone; Nar20, DMN with 20 mg/kg/d naringenin by oral gavage; Nar50, DMN with 50 mg/kg/d naringenin by oral gavage.

Fig. 4. Effects of Naringenin on Liver MDA Content in DMN-Treated Rats for 4 Weeks

DMN was intraperitoneally given at a dose of 10 mg/kg on 3 consecutive days a week for 4 weeks to each group except the control group. Values are mean $\pm$ S.E. of 6 rats. Statistical significance: ** p <0.01 vs. DMN, and ^{###} p <0.001 vs. control, respectively. DMN, DMN alone; Nar20, DMN with 20 mg/kg/d naringenin by oral gavage; Nar50, DMN with 50 mg/kg/d naringenin by oral gavage.

septa in the DMN-treated rats (Fig. 3C2). However, in naringenin-treated groups, only traces of α -SMA positive cells were detected. The level of α -SMA expression was almost the same as that in the liver of the control (Fig. 3C3). Quantitative analysis by an image analysis technique showed a remarkable reduction of α -SMA-positive regions in the naringenin-treated groups compared with the DMN group (Table 3).

Lipid Peroxidation The lipid peroxidation in the liver was measured by MDA determination in liver homogenate. As shown in Fig. 4, the MDA content in DMN group was higher than that of the control group (165% of control) and in the Nar20 and Nar50 groups, it provided 64% and 50% of protection, respectively. These data indicate that naringenin treatment inhibited lipid peroxidation in the DMN-induced liver damage.

Antioxidative Activity of Naringenin Antioxidant activity of naringenin was estimated by examination of the inhibitory effect against $\text{FeSO}_4/\text{H}_2\text{O}_2$ -induced lipid peroxidation on rat liver homogenate. As a positive control, BHT and fisetin were also tested. BHT and fisetin had high inhibitory effect against $\text{FeSO}_4/\text{H}_2\text{O}_2$ -induced lipid peroxidation with IC_{50} values of 5.8 and 11.7 μM , respectively, whereas IC_{50} of naringenin was calculated to be more than 42 μM .

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 proteins. Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation. HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver,^{27,28)} and activated HSCs have been identified as the primary source of excess accumulation of ECM components in liver fibrosis.

In this study, naringenin 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 naringenin 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 naringenin ingestion and that naringenin significantly suppressed the increased collagen accumulated in the DMN-induced liver injury in rats. Taken together, these findings suggest that the antifibrotic effect of naringenin 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.^{29,30)} Much experimental and clinical data indicate that a common link between chronic liver damage and hepatic fibrosis may be related to oxidative stress,^{31,32)} which recently has been reported to be associated with the HSC activation.^{33,34)} Peroxidation of lipids could dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in the pathogenesis of disease. There is sufficient evidence suggesting that lipid peroxidation can occur in both acute and chronic liver injuries. It has been shown that certain lipid peroxidation products induce genetic over-expression of fibrogenic cytokines and increase the synthesis of collagen³⁵⁾ by initiating the activation of HSCs.^{36,37)} Therefore, reducing oxidative stress, which is an important stimulus to activation of HSCs, is a relatively practical avenue of intervention. This study showed that the DMN-treated rats exhibited increased levels of hepatic MDA and that naringenin reduced this increase. These outcomes suggest that the mechanism for the hepatoprotective effects of naringenin in the development of liver fibrosis may be related to the reduction of lipid peroxidation.

Flavonoids, a group of naturally occurring benzo- γ -pyrone derivatives, have been shown to possess several biological properties including hepatoprotective, antithrombotic, anti-inflammatory, and antiviral activities, many of which may be related, partially at least, to their antioxidant and free-radical-scavenging ability.³⁸⁾ However, naringenin showed less antioxidant activity than other flavonoids in iron-dependent lipid peroxidation systems, and did not scavenge the free radical of DPPH.³⁹⁾ A recent study has reported that naringenin protected hepatocytes from *t*-BHP-induced injury,¹³⁾ which might not be directly correlated with the antioxidant activity of naringenin in the system. It is interesting that naringenin, which shows less antioxidant activity, inhibited indirectly lipid peroxidation in the DMN-induced liver damage, although, the mechanism that how naringenin can influence the hepatic MDA levels still remains to be elucidated.

In summary, the present study demonstrated that naringenin 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, naringenin might also produce beneficial effects by reducing oxidative stress in DMN-treated rats, which exerted protective effects against HSC activation. Hepatic fibrosis is a prepathologic state of cirrhosis that occurs as a consequence of severe liver damage in diverse chronic liver diseases. Cirrhosis plays a role in the carcinogenesis of several types of cancer. In particular, the risk of hepatocellular carcinoma was substantially increased in patients with liver cirrhosis.⁴⁰⁾ The prevention of hepatic fibrosis may serve as an important potential target for chemoprevention.⁴¹⁾ Our data suggest that naringenin, one of the most abundant flavonoids in citrus fruits and tomatoes, may be potentially useful in the prevention of the development of hepatic fibrosis.

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