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Effects of naringenin on TGF-β during ethanol induced hepatotoxicity

Jayachitra Jayaraman[1]* and Nalini Namasivayam[2]

[1]*Department of Biochemistry, D.G.G.Arts College for Women, Mayiladuthurai, Tamilnadu, INDIA.*
[2]*Faculty of Science, Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar- 608 002, Tamilnadu, India.*
Corresponding author: **Jayachitra Jayaraman**

ABSTRACT

The hypolipidemic effects of naringenin on liver fibrosis induced by exposure to ethanol in rats are investigated. Rats were divided into four groups, groups 1 and 2 received isocaloric glucose and 0.5% carboxymethyl cellulose (CMC); groups 3 and 4 received 20% ethanol equivalent to 6g/kg body weight every day for the total experimental period of 60 days. In addition, groups 2 and 4 were supplemented with naringenin (50mg/kg p.o) every day for the last 30 days of the experiment. The results showed significantly elevated levels/activities/expression of serum aspartate and alanine transaminases in ethanol fed rats as compared to those of the control. Ethanol fed rats also exhibited increased staining for the presence of transforming growth factor-β (TGF-β) protein adducts in the liver. Supplementation with naringenin for the last 30 days of the experiment to ethanol-fed rats significantly decreased the activities/expression of serum aspartate and alanine transaminases and also decreased staining for the presence of transforming growth factor (TGF-β) protein adducts in the liver as compared to the control rats. These findings suggest that naringenin has a protective effect on liver injury and can inhibit liver fibrosis induced by ethanol in rats. Naringenin improved the histological changes of fibrosis. The mechanism, possibly involves its effect on inhibiting TGF-β and suppressing the activation of hepatic stellate cells.

Keywords: Naringenin; Liver damage; Ethanol; Histochemistry

INTRODUCTION

Ethanol is a powerful inducer of hyperlipidemia. Oxidation of large amounts of alcohol results in the release of excess hydrogen ions, which alter the NAD/NADH ratio and changes the oxidation-reduction potential of liver cells. Ethanol-induced increase in the NAD/NADH ratio is a sign of major change in hepatic metabolism during ethanol oxidation [1]. The redox-related inhibition of fatty acid oxidation and the enhancement of triglyceride synthesis are the main pathogenic mechanisms in the development of alcoholic fatty liver [2]. Accumulation of lipids in the hepatocytes is the most striking manifestation of alcohol-induced liver injury. In chronic lipid accumulation, the liver cells become fibrotic, leading to impaired liver function. Ethanol also causes changes in the metabolism of lipoproteins [1].

Free radical formed on alcohol consumption affects the permeability of hepatocytes, leading to leakage of enzymes such as serum transaminases (AST, ALT), alkaline phosphatase (ALP) [3]. An elevation in the activities of these serum enzymes

is generally regarded as one of the most sensitive markers of liver damage [4].

TGF-β induces collagen synthesis in stellate cells by increasing the production of extracellular matrix proteins and inhibits the synthesis of matrix-degrading proteolytic enzymes. Elevated TGF-β production by kupffer cells was implicated as a trigger for collagen deposition in alcoholic cirrhosis in a rat model. [5, 6] TGF-β is the major fibrogenic cytokine that is elevated during chronic liver disease (CLD) progression, including ALD [7]. In fibrogenic stages of ALD, TGF-β accounts for the activation of hepatic stellate cells (HSC) and ECM production [8]. In addition, TGF-β was shown to mediate hepatocyte plasticity and mesenchymal transition, thus contributing to (Myo) fibroblast populations [9].

TGF-β is also considered to be the main inducer of the myofibroblastic phenotype: it up-regulates α-smooth muscle actin (α-SMA) as well as an ECM protein expression in fibroblasts. [10] Following cholestatic injury the liver undergoes tissue modeling process that combines regeneration and fibrogenesis. During this repair process, the ECM contains large number of alpha-smooth muscle actin (α-SMA) immuno reactive cells known as myofibroblasts: however their origin still remains enigamitic. Cassiman et al., [11] and Ramm et al., [12] demonstrated that the a-SMA immuno positive cells, mainly reside in the portal ducts and fibrous septa and their location corresponds to the distribution of collagen.

Naringenin (4', 5, 7-trihydroxyflavanone) (Fig. 1) Is a predominant flavonone abundant in fruits such as grapes, tangelo, blood orange, lemons, pummelo and tangerines [13]. Naringenin is the main metabolite of naringin which is the important flavonoid in *Exocarpium citri grandis*. Naringenin is used as a traditional medicine in China [14]. It has been reported to have several biological effects such as anticancer [15], antimutagenic [16], anti-inflammatory [17] antiatherogenic [18] and antifibrogenic [19] properties. Daily intake of citrus flavonoids has been estimated to be approximately 68g on an average in the USA, mainly ingested via fruit juices.

Thus, our present investigation was carried out to study the effect of naringenin on ethanol induced alterations in the hepatic fibrotic markers in male wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

Naringenin was purchased from Sigma Chemical Co (St. Louis, MO, USA). Ethanol was obtained from E.I.D Parry India Ltd. (Nellikuppam, Cuddalore District, South India). All other chemicals used were of analytical grade and were obtained from Central Drug House Private, Ltd, Mumbai. Anti-TGF-β antibody (Mouse monoclonal Ab, Code no. NCL-TGF-β) was from Novacastra, UK. The Peroxidase - polymer kit was from Biogenix Life Science Ltd, USA.

Animals

Adult male albino Wistar rats (150-170g) were assayed from the Central Animal House, Rajah Muthiah Medical College and Hospital, (RMMC&H), Annamalai University. The rats were housed in plastic cages under controlled conditions of 12-h light-dark cycle, 50% humidity and temperature of 28°C. They were all fed a standard pellet diet (Lipton Lever Mumbai, India) and water *ad libitum*. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (registration no: 160/1999/CPCSEA/557) and animals were cared for in accordance with the Indian National Law on animal care and use.

Study design

Animals were divided into four groups of 8 rats each and all were fed the standard pellet diet. Rats in groups 1 and 2 received isocaloric glucose from a 40% glucose solution and 0.5% CMC. Animals in groups 3 and 4 received 20% ethanol (equivalent to 6g/kg body weight) as an aqueous solution by intragastric intubation for 60 days as described previously [20]. At the end of this period, the dietary protocol of group1 and 3 animals was unaltered. However, group 2 animals received naringenin (50mg/kg bodyweight/day) suspended in 0.5% CMC for the next 30 days, and group 4 animals continued to receive ethanol every day along with naringenin as in group 2 for the next 30 days. The total experimental duration was 60 days. The study design is shown in Figure 2.

The animals were then fasted overnight, anesthetized with an intramuscular injection of ketamine hydrochloride (30mg/kg) and blood samples were collected by retro-orbital puncture.

Blood samples were collected in heparinized tubes and centrifuged for the separation of plasma.

Biochemical estimations

Serum AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were assayed using a diagnostic kit based on the method of Reitman and Frankel. [21].

Immunohistochemistry

For immunohistochemistry, 4 μm tissue sections were deparaffinized and incubated with peroxide blocking reagent, power block solution for 10 min. Nonspecific adsorption was minimized by leaving the sections in 3% bovine serum albumin in PBS for 30 min. Sections were incubated overnight with a 1:50 dilution of anti-TGF antibody (Mouse monoclonal antibody, Novacastra, UK). The sections were then rinsed well with phosphate buffer and incubated with super enhancer reagent for 30 min. After rinsing with phosphate buffer, incubation was done with peroxidase polymer kit for 30 min. After washing thoroughly with phosphate buffer, the sections were incubated with diaminobenzidine (DAB) substrate solution for 5 min. Sections were counterstained with hematoxylin and observed under light microscopy. All the sections from the various groups were incubated under the same conditions with similar antibody concentrations, and run simultaneously, in order to make the immunostaining comparison among the different experimental groups.

Statistical analysis

Data were analyses by one way analysis of variance followed by Duncan's multiple range test using SPSS for Windows (v. 11.0; SPSS Inc., Chicago, IL, USA). Results are presented as means ± SD of eight rats in each group. Values of $P < 0.05$ were regarded as statistically significant and the data are represented as mean ± SD for the absolute values or percent of controls as indicated in the vertical axis legends of figures. The statistical significance of differential findings between the experimental groups and control was determined.

RESULTS

Effect of naringenin and ethanol on liver marker enzymes

Table 1 shows the activities of serum AST and ALT. The activities of both the enzymes were significantly increased in ethanol fed rats as compared to the control rats. Supplementation with naringenin to ethanol-fed rats (group 4) significantly decreased the liver marker enzymes as compared to the unsupplemented ethanol fed rats (group 3; $P < 0.05$).

Effect of naringenin and ethanol on TGF-β

Figure 3, illustrates the immunohistochemistry of TGF-β was localized in the cytoplasm. The control rats exhibited mild TGF-β protein in the liver. Control rats treated with naringenin showed a similar pattern of TGF-β as seen in control. Ethanol treated rat liver increased TGF-β positive staining around the central vein area. Supplementation with naringenin to ethanol treated rats showed reduced TGF-β protein and reduction in fibrosis.

Figure: 1

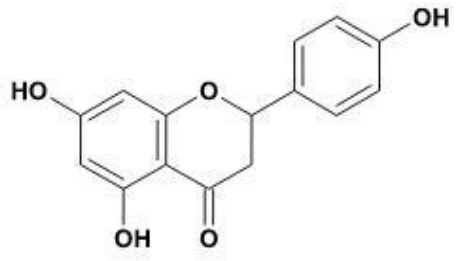

Figure: 1 structure of naringenin (4', 5,7-trihydroxyflavonone)

Figure: 2

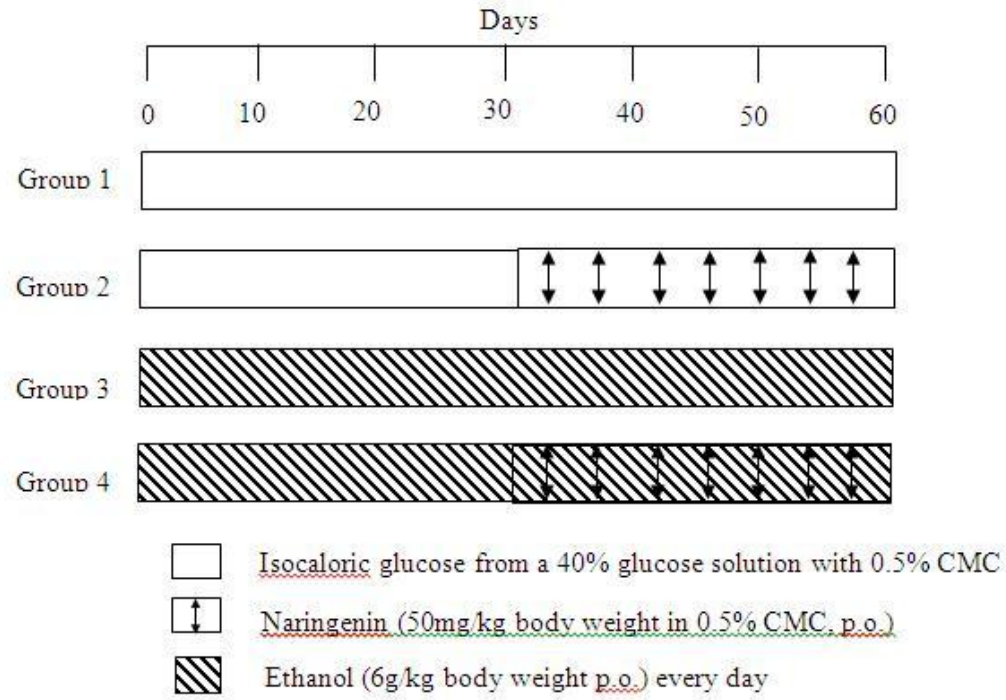

Figure: 2 Diagrammatical representation of the experimental protocol

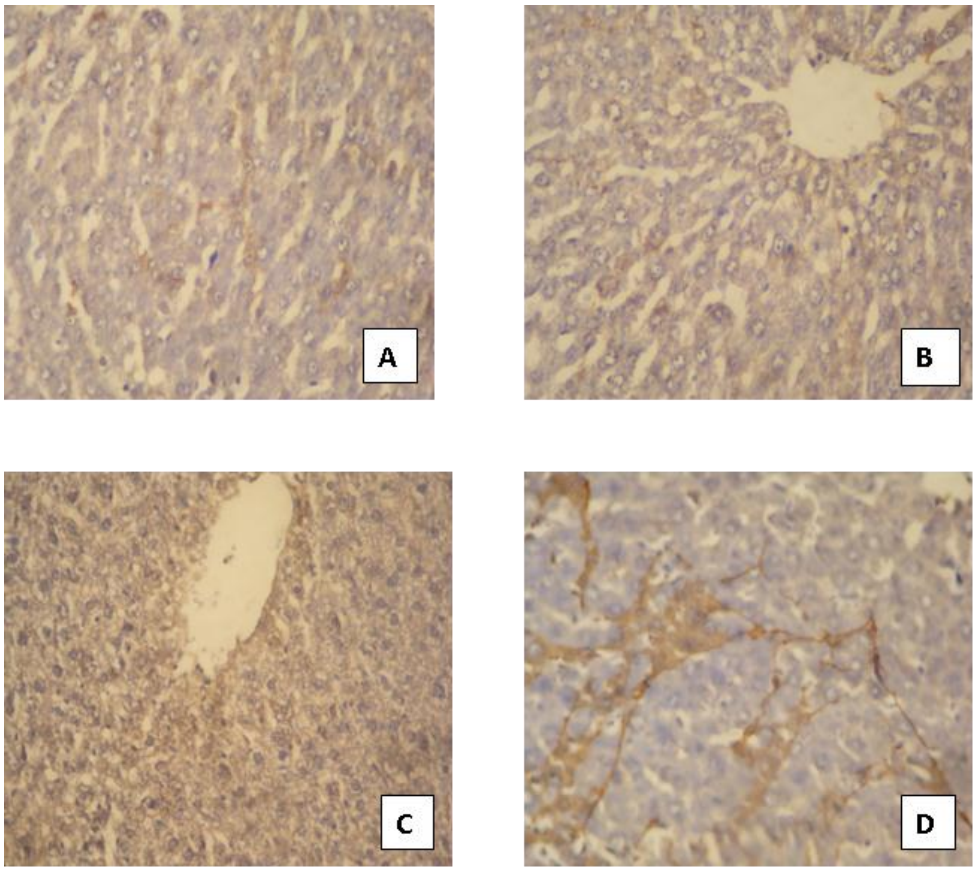

Figure: 3 Immunohistochemical staining of liver TGF-β tissue (40X)

Fig. A: Normal control rat liver

Shows no histological alterations displayed normal pattern

Fig. B: Naringenin treated rat liver

Shows no histological alteration

Fig. C: Ethanol treated rat liver

The liver sections of ethanol fed rats exhibited an increase in collagen content and displayed bundles of collagen fibers surrounding the lobules, massive deposition of collagen around central vein and portal triad.

Fig. D: Ethanol + naringenin treated rat liver

Shows the decreased the collagen content, reduced the bundles of collagen fibers, minimized collagen deposition around the central vein, portal triad and also reduced the scores of liver fibrosis.

Table 1 Effect of naringenin and ethanol on hepatic marker enzymes of control and experimental rats

Groups	Control	Control + Naringenin	Ethanol	Ethanol + Naringenin
Plasma				
Aspartate transaminase (IU/L)	79.84±7.68^{a}	82.13±7.90^{a}	112.40±10.81^{b}	87.27±8.40^{a}
Alanine transaminase (IU/L)	28.86±2.77^{a}	30.81±2.96^{a}	60.38±5.81^{b}	32.76±3.15^{a}

Values are mean ± S.D. of eight rats in each group. Values not sharing a common superscript letter differ significantly at p< 0.05 (Duncan's multiple range test).

Table 2. Effect of naringenin and ethanol on serum and tissue phospholipids of control and experimental rats

Groups	Serum(mg/dL)	Liver	Kidney	Heart
Phospholipids (mg/100g tissue)				
Control	88.8±8.55^{a}	1765.1±169.9^{b}	1449.0±139.4^{b}	87.27±8.40^{a}
Control+ Naringenin	87.8±8.45^{a}	1825.3±175.7^{b}	1516.8±146.0^{b}	32.76±3.15^{a}
Ethanol	127.6±12.28^{b}	1265.4±121.8^{a}	1213.8±116.8^{a}	1528.3±147.1^{b}
Ethanol+ Naringenin	1033.8±99.5^{b}	1038.5±99.9^{b}	731.54±70.41^{a}	1021.22±98.30^{b}

Values are mean ± S.D. of eight rats in each group. Values not sharing a common superscript letter differ significantly at p< 0.05 (Duncan's multiple range test).

DISCUSSION

Chronic consumption of ethanol is known to cause injury to hepatocytes. The elevated activities of the serum enzymes such as AST and ALT observed in alcohol-fed rats may indicate increased permeability, damage or necrosis of hepatocytes [22]. In our study, chronic ethanol consumption caused a significant increase in the activities of AST and ALT, which could be due to severe damage to the liver cell membrane. The reduced activities of these serum enzymes on naringenin supplementation of ethanol-fed rats indicates the hepatoprotective potential of naringenin.

Activated Kupffer cells produce various mediators, including cytokines, eicosanoids, proteases, and oxygen radicals that participate in inflammation, immune responses, and modulation of hepatocyte metabolism [23]. In our study, increased TGF-β expression was noted in the liver of ethanol fed rats. Transforming growth factor ((TGF-β) is a critical cytokine important in fibrogenes than in inflammation [24]. Increased level Jayachitra Jayaraman amil s of TGF-β was reported in isolated rat Kupffer cells after 10 weeks of treatment with ethanol and high-fat diet [25]. Kamimura and Tsukamoto, [26] also reported that the mRNA expression TNF-α, IL-6, and TGF-β1 increases after 17 weeks of treatment with ethanol and high-fat diet in isolated Kupffer cells. Increased TGF-β expression may be due to inflammation, necrosis and oxidative stress.

Supplementation with naringenin effectively decrease the TGF-β expression in the liver of ethanol fed rats. Further, Liu et al., [27] showed that naringenin could reduce the TGF-β1-induced accumulation of ECM in cultured HSC-T6 cells. Decreased TGF-β expression may be due to attenuated inflammation, necrosis and reduced oxidative stress.

CONCLUSION

The results of the study demonstrate the potential beneficial effects of naringenin on alcoholic liver damage. The effect of Naringenin against ethanol induced toxicity by modulating the expression of transforming growth factor-β (TGF-β) and lipid changes.

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