Antifibrotic effects of green tea on *in vitro* and *in vivo* models of liver fibrosis

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AIM: To examine the protective effect of green tea extract (GT) on hepatic fibrosis *in vitro* and *in vivo* in dimethylnitrosamine (DMN)-induced rats.

METHODS: HSC-T6, a rat hepatic stellate cell line, was used as an *in vitro* assay system. Cell proliferation, collagen content, and type I collagen expression were examined in activated HSC-T6 cells. Collagen was determined by estimating the hydroxyproline content. In rats with DMN-induced hepatic fibrosis, serum acetaldehyde, alanine aminotransferase and aspartate aminotransferase activities, liver hydroxyproline and lipid peroxides were examined. Pathologic changes were examined by hematoxylin & eosin staining.

RESULTS: GT administration prevented the development of hepatic fibrosis in the rat model of DMN-induced liver fibrosis. These results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of liver collagen deposition. Accordingly, inhibition of proliferation, reduced collagen deposition, and type I collagen expression were observed in activated HSC-T6 cells following GT treatment. These results imply that GT reduced the proliferation of activated HSC and down regulated the collagen content and expression of collagen type 1, thereby ameliorating hepatic fibrosis.

CONCLUSION: This study demonstrates that green tea administration can effectively improve liver fibrosis caused by DMN, and may be used as a therapeutic option and preventive measure against hepatic fibrosis.

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Key words: Dimethylnitrosamine; Green tea extract; HSC-T6 cell; Liver fibrosis; Rat model; Type 1 collagen

INTRODUCTION

Hepatic fibrosis is a consequence of severe liver damage and occurs in many forms of chronic liver damage, including virus infection, autoimmune liver diseases and sustained alcohol abuse. Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis. The key cellular and molecular events involved in the pathogenesis of liver fibrosis include activation of HSC to a myofibroblast-like phenotype, production of excess matrix proteins, and increased cell proliferation. Overproduction of extracellular matrix (ECM) components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis. Therefore, suppression of HSC activation has been proposed as a therapeutic target against hepatic fibrosis.

Acetaldehyde, a highly reactive compound produced by alcohol metabolism, stimulates the deposition of ECM proteins. Acetaldehyde also stimulates type I collagen synthesis and gene transcription in cultured rat and human HSC and in human liver fibroblasts.

Several studies have shown that lipid peroxidation stimulates collagen production in fibroblasts and HSC, and plays an important role in the development of liver fibrosis.
fibrosis. Lipid peroxidation has been shown to stimulate the expression of collagen gene transcripts[^6]. It has recently been shown that stellate cells are activated by free radicals as well as by malondialdehyde (MDA), a product of lipid peroxidation[^9]. In addition, stellate cell activation by type 1 collagen has been shown to be blocked by antioxidants[^8], suggesting that lipid peroxidation may play a role in hepatofibrogenesis.

Green tea, which is a widely consumed drink, has received much attention due to its beneficial biological effects. Polyphenols, often collectively referred to as catechins, account for up to 30% of the dry weight and serve as a major effective component of green tea. The effects of green tea have been widely studied and antioxidant, antiallergic, antimutagenic/anticarcinogenic, and antibacterial effects have been documented[^10^-^12]. It has been shown that an aqueous extract of polyphenols from green tea (Camellia sinensis) reduces liver fibrosis in rats induced by bile duct ligation, and epigallocatechin gallate (EGCG), the major component in green tea, was implicated as the main active ingredient[^13]. EGCG has been reported to suppress cell proliferation and collagen production in HSC[^14]. In addition, the hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies[^15^-^17]. However, the hepatoprotective effect of green tea in dimethylnitrosamine (DMN)-induced models has not been studied. The DMN-induced liver fibrosis model can reproduce most of the features observed during human liver fibrosis[^17]. Furthermore, this model has other advantages such as progressive and remarkable pathological alterations, a high fibrosis reproduction rate, and a low mortality rate in experimental animals[^17]. This model is also stable even after termination of DMN administration and is a reliable tool for screening antifibrotic agents[^18]. Therefore, the aim of the present study was to examine the protective effect of green tea extract (GT) on hepatic fibrosis in a rat HSC line and in a rat model of DMN-induced hepatic fibrosis.

### MATERIALS AND METHODS

#### Preparation of GT

Green tea, cultivated from Cheju island, Korea, was extracted with 80% methanol and freeze-dried.

#### In vitro experiment

**Cell culture:** HSC-T6 cells, an immortalized rat HSC line, were cultured in Dulbecco’s minimal essential medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and 0.5% antibiotics. Cultures were placed in a humidified atmosphere of 5% CO2 with 10% FBS (Gibco) and 0.5% antibiotics. Cultures were incubated at 37°C in 5% CO2 with 10% FBS (Gibco) and 0.5% antibiotics. The medium was changed twice a week. Acetaldehyde (50 μg/mL) were also added to increase the collagen proline hydroxylation and to prevent collagen cross-linking. After 24 h of treatment with GT (100 μg/mL), aliquots of medium were transferred into immunowell plates, and glutaraldehyde (0.01%) was added and incubated at room temperature for 1 h. Collagen type I antibody (1:4000, Abcam Co., Cambridge, UK) was added and further incubated for 2 h at 37°C. The antigen-coated plates were blocked with casein and incubated with the secondary antibody (1:8000) linked to peroxidase, and subsequently re-incubated with substrate (TMB 10 mg/mL, 3% H2O2, 50 mmol/L sodium acetate buffer, pH 5.1) for 15 min. The enzymatic reaction was stopped by adding 1 mol/L H2SO4, and the absorbance at 450 nm was measured using a microplate reader.

**Expression of collagen type 1:** The expression of collagen type 1 was observed by ELISA. HSC-T6 cells, seeded on 24-well plates at a density of 1.5 × 10^4 and cultured until 90% confluency, were treated with serum-free DMEM with or without 175 μmol/L acetaldehyde. Ascorbic acid (50 μmol/L), and 3-aminopropionitrile fumarate (100 μg/mL) were also added to increase the collagen proline hydroxylation and to prevent collagen cross-linking. After 24 h of treatment with GT (100 μg/mL), aliquots of medium were transferred into immunowell plates, and glutaraldehyde (0.01%) was added and incubated at room temperature for 1 h. Collagen type I antibody (1:4000, Abcam Co., Cambridge, UK) was added and further incubated for 2 h at 37°C. The antigen-coated plates were blocked with casein and incubated with the secondary antibody (1:8000) linked to peroxidase, and subsequently re-incubated with substrate (TMB 10 mg/mL, 3% H2O2, 50 mmol/L sodium acetate buffer, pH 5.1) for 15 min. The enzymatic reaction was stopped by adding 1 mol/L H2SO4, and the absorbance at 450 nm was measured using a microplate reader.

**In vivo experiment**

**Animals and treatments:** Male albino rats (235-250 g) were purchased from Samtako (Kyunggi-do, Korea) and housed in controlled temperature and relative humidity, and a 12 h light/dark cycle. All experiments were performed according to National guidelines for the use of animals in biomedical research. The rats were randomly assigned to four groups of eight rats each: the normal control group without any treatment (NC), the hepatic fibrosis control group (FC), and hepatic fibrosis with 100 mg/kg GT treated group (FG). Hepatic fibrosis was induced by intraperitoneal injections of 10 mg/kg dimethylnitrosamine (DMN, Sigma, St. Louis, USA) for 3 consecutive days each week over a period of 4 wk. Normal saline was given to NC rats. GT was administered in drinking water which was calculated according to the amount of water consumed the previous day. At the end of the 4 wk experimental period, all rats were killed under ether anesthesia. Blood was obtained from the...


Hepatotoxicity and lipid peroxidation: Hepatotoxicity was assessed by quantifying the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a spectrophotometric diagnostic kit (Youngdong Pharmaceutical Co., Korea). Lipid peroxidation in the liver and serum were determined by measuring the levels of MDA, an end product of lipid metabolism. For the serum sample, 3 mol/L sulfuric acid and 100 g/L phosphotungstic acid were added and incubated at room temperature for 10 min, and then centrifuged. For the liver sample, homogenates of liver in potassium phosphate buffer were prepared. MDA contents in the serum and liver samples were determined using a colorimetric reaction with thiobarbituric acid.

Hepatic hydroxyproline content: A portion of liver tissue (200 mg) was homogenized in 10 volumes of 0.5 mol/L potassium phosphate (KP) buffer and hydroxyproline content was measured as described above.

Histology of liver: Liver tissues were fixed in 10% neutral buffered formalin, dehydrated with 50%-100% ethanol, and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin-eosin. The control group showed normal architecture while acetaldehyde and GT treatment for 24 h. Treatment with 100 μg/mL GT significantly reduced cell hydroxyproline content by 23.0% ± 2.1% compared to the control group. Furthermore, the expression of type 1 collagen was up-regulated by acetaldehyde stimulation, and GT markedly reduced collagen type 1 expression in a dose-dependent manner. Acetaldehyde at a concentration of 175 μmol/L induced collagen type 1 expression by 17.4% ± 0.1%, and 10, 50 and 100 mg/mL GT reduced collagen type 1 expression by 15.2% ± 2.2%, 15.5% ± 1.3%, and 23.0% ± 1.1%, respectively (Figure 1).

Serum biochemical analysis
AST and ALT concentrations in serum were used as biochemical markers to evaluate hepatic injury. ALT is a cytosolic enzyme, primarily present in the liver. An increase in plasma ALT indicates liver damage more specifically than AST. AST, which is a mitochondrial enzyme present in large quantities in the heart, liver, skeletal muscle, and kidney, in part indicates liver injury. Serum activities of ALT and AST were markedly increased with DMN treatment and GT supplementation attenuated the elevation of AST and ALT activities (Table 1).

Histology and fibrosis marker
Liver fibrosis was evaluated by hematoxylin & eosin staining. The control group showed normal architecture
(Figure 2A), whereas the DMN-treated group exhibited necrosis, congestion, hemorrhage, and destruction of the lobular architecture (Figure 2B). Red blood cells from blood vessels were found in liver tissue due to the collapse of the matrix structure. GT administration exhibited notable recovery effects (Figure 2C).

**Hydroxyproline and lipid peroxide content in liver**

The histological findings were corroborated by biochemical parameters of liver tissue collagen content determined by hydroxyproline, and lipid peroxide determined by MDA.

Hydroxyproline, a product of collagen metabolism, is an amino acid characteristic of collagen. The total collagen present in liver was, therefore, determined by estimating the hydroxyproline content. As shown in Figure 3, hydroxyproline content was significantly increased following DMN treatment (FC), indicating that the liver fibrosis model was successfully established. GT administration (FG, 100 mg/kg) restored the hydroxyproline content in fibrotic liver. Lipid peroxides, measured in terms of the formation of MDA, were significantly increased in DMN-induced rat liver. GT administration significantly reduced the lipid peroxide level.

**DISCUSSION**

Hepatic fibrosis is characterized by an abnormal accumulation of ECM proteins, particularly collagen. When hepatic fibrosis occurs, collagen proliferation, mainly collagen type 1 and 3, accounts for 50% of the total protein in fibrotic liver, and collagens are the main components of ECM. Therefore, collagen type 1 is an important parameter reflecting the metabolism of collagen in liver. The main collagen producing cells in the liver are HSC, which proliferate and undergo a process of activation during the development of fibrosis resulting in increased capacity for collagen synthesis. Changes in hydroxyproline content in the liver are considered an index for collagen metabolism and provide valuable information on the biochemical and pathological states of liver fibrosis. The present study demonstrated that consumption of GT prevented the development of hepatic fibrosis in a rat model of DMN-induced liver fibrosis. The results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in liver.

Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC-T6 cells following GT treatment. Activated HSC are the main source of ECM when liver fibrosis occurs. Therefore, these results imply that GT inhibit the proliferation of activated HSC and down regulate the collagen content and expression of collagen type 1, thereby inhibiting hepatic fibrosis. The results of the present study are consistent with previous observations showing that EGCG, the major component in green
Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis. Overproduction of extracellular matrix components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis.

Research frontiers

Hepatoprotective effects of green tea against carbon tetrachloride, cholesterol and alcohol induced liver fibrosis were reported in many studies. However, the hepatoprotective effect of green tea in dichloroacetic acid (DCA)-induced models has not been studied.

Innovations and breakthroughs

The present study demonstrates that consumption of green tea prevents the development of hepatic fibrosis in a rat model of DCA-induced liver fibrosis. These results were confirmed by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in the liver. Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC cells following green tea treatment.

Applications

This study demonstrates that green tea may protect liver cells and reduces the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

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