Genistein decreases liver fibrosis and cholestasis induced by prolonged biliary obstruction in the rat

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Abstract

Fibrosis accompanies most chronic liver disorders and is a major factor contributing to hepatic failure. Therefore, the need for an effective treatment with the aim of modifying the clinical course of this disease is evident. The aim of this work is to determine whether genistein, which has been shown to modulate the physiology and pathophysiology of liver, is able to decrease experimental liver fibrosis and cholestasis. In male Wistar rats, the common bile duct was ligated. Administration of genistein (5 µg rat⁻¹, day⁻¹, p.o.) began four weeks after biliary obstruction and continued for a further four weeks. The liver was used for histological and ultrastructural analysis and for collagen quantification (hydroxyproline content). The degradation of Matrigel® and collagen type I was determined in homogenized liver. Bilirubins and enzyme activities were measured in serum. Genistein was able to improve normal liver histology, ultrastructure, collagen content, and biochemical markers of liver damage. It also increased Matrigel® and collagen type I degradation. In summary, the present report shows that genistein inhibits the fibrosis and cholestasis induced by prolonged biliary obstruction in the rat. Genistein has therapeutic potential against liver fibrosis.

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Introduction

Liver fibrosis results from chronic damage to the liver in conjunction with the accumulation of extracellular matrix proteins, which is a characteristic of most types of chronic liver diseases. 1-4 The main causes of liver fibrosis in industrialized countries include chronic HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH). The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines cirrhosis. 5,6

Hepatic fibrosis is considered a model of wound-healing response to chronic liver injury, and it is characterized by the activation of hepatic stellate cells (HSCs). The activation of HSCs involves the transdifferentiation from a quiescent state into myofibroblast-like cells with the appearance of smooth muscle α-actin (SMA) and loss of cellular vitamin A storage. 7 The activated HSCs are distinguished by accelerated proliferation and enhanced production of ECM components. Cross-talks between parenchymal and nonparenchymal cells constitute the major interactions in the development of hepatic injury and fibrosis. Soluble factors, such as cytokines, chemokines, or reactive oxygen species are the mediators in these cross-talks, and are possible targets for therapeutic consideration. 8

Genistein (4,5,7-trihydroxyisoflavone), a soy-derived isoflavone, has recently attracted much attention in the medical scientific community. Genistein is a potent protein tyrosine kinase inhibitor that attenuates growth factor- and cytokine-stimulated proliferation of both normal and cancer cells. 9 Extensive epidemiological, in vitro, and animal studies have been performed, and most studies indicated that genistein has beneficial effects on a multitude of human disorders, including cancers, cardiovascular diseases, osteoporosis, and postmenopausal symptoms. 10-12 The role of genistein in the physiology and pathophysiology of liver has been studied in the last decade. 13-19 More than a dozen reports regarding the effect of genistein on HSCs have appeared. Antifibrotic effects of genistein in vitro have been shown. 20,21 However, at present there are no reports regarding the effect of
genistein on fibrogenesis in vivo. Therefore, the aim of
this study was to evaluate the effect of genistein on the fi-
brosis and cholestasis induced by prolonged biliary ob-
struction in the rat.

Materials and methods

Animal treatment and biliary obstruction

Male Wistar rats weighing 200 g were used. Animals
had free access to food (Standard Purina Chow; St Louis,
MO) and water. Obstructive jaundice was induced by dou-
ble ligation and sectioning of the common bile duct. Con-
trol rats were sham operated. Genistein (Sigma Chemical
Co., St Louis, MO) was dissolved in water and adminis-
tered at a dose of 5 µg per rat through an intragastric tube.
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This chosen dose was based on previous studies by our
group.24 Administration of genistein began four weeks af-
after biliary obstruction and was continued for a further four
weeks. Another group of animals was bile duct ligated, but
received only water, instead of the drug (fibrosis group).
Each group consisted of six rats. The animals were sacri-
ficed eight weeks after surgery under light ether anesthe-
sia; blood was collected by heart puncture, and the liver
was rapidly removed. Small liver sections fixed in Bouin’s
medium were used for trichromic staining for histological
examination under light microscopy. This investigation
followed the Guiding Principles in the Care and Use of
Animals from the National Institutes of Health.25

Collagen quantification

Collagen concentration was determined by measuring
hydroxyproline content in fresh liver samples after diges-
tion with acid.26 The procedure was as follows. Fresh liver
samples (100 mg) were placed in ampoules, 2 ml of 6 N
HCl was added, and the ampoules were sealed and hydro-
lyzed at 100 °C for 48 h. The samples were then evaporated
at 50 °C for 24 h and resuspended in 3 ml of sodium ac-
etate/citric acid buffer (pH 6.0); 0.5 g of activated charcoal
was added, and the mixture was stirred vigorously then
centrifuged at 5000 × g for 10 min. The mixture was kept
for 20 min at room temperature and the reaction was
stopped by the addition of 2 M sodium thiosulfate and 1
N sodium hydroxide. The aqueous layer was transferred to
test tubes. The oxidation product from hydroxyproline
was converted to pyrrole by boiling the samples. The pyr-
role-containing samples were incubated with Ehrlich’s re-
agent for 30 min and their absorbance was read at 560 nm.
Recovery of known amounts of standards was carried out
on similar liver samples to provide calibration samples.

Tissue extraction of proteases

Liver samples were homogenized with 10 volumes of
buffer (0.075 M) potassium acetate, 0.3 M NaCl, 0.01 M
EDTA, 0.1 M L-arginine and 0.25% Triton X-100; pH 4.2)
on ice. After being kept in ice water for 3 h, the homoge-
nized samples were centrifuged at 12 000 × g and 4 °C for
10 min. Supernatants were stored at -70 °C until assay.

Proteolytic activity assays

Assay plates were prepared by diluting ice-cold col-
lagen I in 0.2% acetic acid with an equal volume of neu-
tralizing buffer (100 mM Tris–HCl, 200 mM NaCl, 0.04% NaN3, pH 7.8) to give a final collagen concentra-
tion of 700 µg/mL. Aliquots (50 µL) of the diluted col-
lagen were added quickly to microwell modules and in-
cubated at 30 °C for 40 h (16 h in a humidified atmos-
phere, then 24 h in a dried atmosphere) to gel and dry
the collagen. The same procedure was performed using
Matrigel® basement membrane extract. Aliquots of
100 µg were used per well and dried overnight after poly-
merization at room temperature.

The enzymatic essay was performed as described by
Nethery et al.27 Enzyme samples were brought to assay
temperature (35 °C) and replicate aliquots (100 µL) were
added to the protein-coated wells. Microwells containing
samples were incubated for 2 h in a humidified container
equilibrated at assay temperature. The samples were de-
canted from the wells, which were washed twice in quick
succession with assay buffer (50 mM Tris–HCl, 100 mM
NaCl, 10 mM CaCl2, 0.02% NaN3; pH 7.5). Stain solution
(100 µL of 0.25 g Coomassie Blue R250, 10% acetic
acid, 50% methanol) was incubated in the wells for 25
min at 25 °C. After the stain was drained off, the wells
were washed three times with distilled water and left to
dry at room temperature. The absorbance at 590 nm was
measured using a Titertek Multiscan automatic spectro-
photometer (Flow Laboratories, Titertek, Huntsville, AL)
with the instrument blank set on unused microwells. Col-
lagenase and urokinase were used as standard samples.

Serum enzyme activities and bilirubins

Serum was obtained for the following determinations:
the activities of alkaline phosphatase, alanine amino
transferase, γ-glutamyl transpeptidase, and for bilirubin
content (Kit Merck, México)

Electron microscopic analysis

For ultrastructural analysis, liver blocks of ca 0.5–0.7
mm3 were fixed with 2.5% glutaraldehyde in 0.1 M phos-
phate buffer (pH 7.2) for 1 h and 1% OsO4 was added to
continue fixing (30 min at 27 °C and 30 min at 4 °C). Af-
ter dehydration with increasing concentrations of ethyl
alcohol, the blocks were embedded in epoxy resin. Ul-
trathin sections (80 nm) were cut and then examined with
a Jeol-200 EX electron microscope at an accelerating
voltage of 80 kV.
Statistical analysis

Data are reported as means ± standard deviation of three independent experiments conducted in quadruplicate. Statistical analysis was performed using a non-parametric ANOVA. Individual differences between treatments was analyzed by a Tukey’s test. Significant differences were established at p < 0.001.

Results

Figure 1 shows the histological analysis of liver sections. Prolonged biliary obstruction was accompanied by an increase in collagen deposition around the portal triad (Figure 1C). In addition, the normal architecture was lost and a marked ductular proliferation was observed. Genistein treatment for four weeks in bile duct-ligated rats restored the normal architecture of the liver. A significant decrease in the collagen content was observed in the bile duct-ligated rats treated with genistein (Figure 1D).

Serum markers of cholestasis and liver injury are shown in Table I. Total bilirubins increased more than 50-fold compared with controls. Serum activities of alkaline phosphatase, alanine aminotransferase, and γ-glutamiltranspeptidase increased approximately three-, six-, and sixfold, respectively, compared to the control group (p < 0.001). Genistein administration to bile duct-ligated rats significantly reduced the markers of cholestasis and liver injury.

Liver collagen content was estimated in liver samples by measuring hydroxyproline. Biliary obstruction induced a sixfold increase in liver collagen content. Treatment of bile duct-ligated animals with genistein significantly decreased the collagen content; p < 0.001 (Figure 2).

Degradation of collagen type I is shown in Figure 3. Bile duct ligation increased twofold the degradation of

![Figure 1](image_url)

Figure 1. Histological study of liver sections from rats treated with genistein. Liver sections from: (A) Control (Sham operated) rats; (B) Genistein; (C) bile duct-ligated rats; (D) bile duct-ligated rats and treated with genistein. Liver tissue were stained with Masson trichrome, collagen can be recognized by blue staining (200 X).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total bilirubins (µmol/L)</th>
<th>AP (µmol/L/min)</th>
<th>ALT (µmol/L/min)</th>
<th>γ-GTP (µmol/L/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>2.5 ± 0.8</td>
<td>45 ± 9</td>
<td>32 ± 6</td>
<td>11.8 ± 8</td>
</tr>
<tr>
<td>Genistein</td>
<td>1.9 ± 1.0</td>
<td>58 ± 15</td>
<td>26 ± 10</td>
<td>9.5 ± 10</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>145 ± 36 b</td>
<td>167 ± 43 b</td>
<td>196 ± 25 b</td>
<td>76 ± 16 b</td>
</tr>
<tr>
<td>Fibrosis + Genistein</td>
<td>45 ± 12 b,c</td>
<td>67 ± 17 c</td>
<td>95 ± 18 b,c</td>
<td>20 ± 9 c</td>
</tr>
</tbody>
</table>

*a Results are expressed as the mean value of experiments performed in duplicated assays with samples from six animals ± SEM.

*b p < 0.05 vs Sham group

c p < 0.05 vs Fibrosis group
Figure 2. Liver collagen content determined in sham-operated rats (sham), genistein treated rats, bile duct-ligated rats (fibrosis) and bile duct-ligated rats and treated with genistein (fibrosis +genistein). Each bar represents the mean ± SEM in experiments performed in duplicate. All groups consisted of six animals. * Means different from the Sham group (p < 0.001). # means different from the fibrosis group (p < 0.001).

Figure 3. Type I collagen degradation by liver homogenates determined in sham-operated rats (sham), genistein treated rats, bile duct-ligated rats (fibrosis) and bile duct-ligated rats and treated with genistein (fibrosis +genistein). Each bar represents the mean ± SEM in experiments performed in duplicate. All groups consisted of six animals. * Means different from the Sham group (p < 0.001). # means different from the fibrosis group (p < 0.001).

Figure 4. Quantitative Matrigel® degradation by liver homogenates determined in sham-operated rats (sham), genistein treated rats, bile duct-ligated rats (fibrosis) and bile duct-ligated rats and treated with genistein (fibrosis +genistein). Each bar represents the mean ± SEM in experiments performed in duplicate. All groups consisted of six animals. * Means different from the Sham group (p < 0.001). # means different from the fibrosis group (p < 0.001).

Figure 5. Electron micrograph of livers from: (A) Control (Sham operated) rats; (B) Genistein; (C) bile duct-ligated rats; (D) bile duct-ligated rats and treated with genistein. HSCs = Hepatic stellate cells; H: hepatocytes, n: nucleus, cf: collagen fibers, (M1500).
genistein restored the ultra structure of hepatocytes which correlated with the improvement of liver function (panel D).

Discussion

Hepatic fibrosis is a dynamic process resulting from chronic damage up to cirrhosis, characterized by accumulation of ECM components in the liver, caused by both markedly increased and unbalanced degradation of connective tissue components. Previous reports have shown that bile duct ligation in the rat for four weeks produces cirrhosis and a sixfold increase in liver collagen content. Our results show that genistein is able to ameliorate the cholestasis, fibrosis, liver architecture, and biochemical markers of liver damage when administered to rats that had been bile duct ligated for four weeks. The mechanism of the action of this compound is probably associated with its ability to reduce the proliferation of HSCs and then the production of hepatic collagen, as well as by increasing matrix degradation; however, the possibility of other actions cannot be disregarded.

The major obstacle to antifibrotic drug development is the slow evolution of fibrosis, which takes years or even decades in humans. Consequently, there is an evident need for an effective treatment with the aim of modifying the clinical course of this disease. A recent insight into the molecular pathogenesis of hepatic fibrosis and the role of activated HSCs provides hope for future development of successful therapy. Genistein is a new drug with hepatic protective properties that may be beneficial in liver fibrosis.

Fibrosis is a well-known phenomenon that leads to loss of normal architecture and function. Thus, restoration of liver homeostasis (i.e., regulation of serum bilirubins and enzymes) by genistein could be explained, at least in part, by the antifibrotic effect of this compound. However, the amelioration of serum bilirubins and enzyme activities cannot be fully explained with the present data. One possibility is that genistein affected the serum bilirubin and enzyme concentration by mechanisms other than by preventing fibrosis. These mechanisms could include decreased production of bilirubins and enzymes or their increased elimination via an antioxidative pathway.

Natural flavonoids possess reactive phenolic groups and show antioxidative properties in vitro. Aneja et al. showed that the pretreatment of animals with genistein markedly increases the intracellular reduced glutathione (GSH) levels in animals treated with CCl₄ and restores them to normal levels. They suggest that the induction of GSH levels may be due to the enhancement of GSH synthesizing enzymes such as c-glutamyl cysteine synthetase and GSH synthetase, which are key enzymes in its biosynthesis. They also speculate that genistein may cooperate with physiological defense molecules such as reduced glutathione in such a way as to protect animals against oxidative stress. Recently, Lee et al. reported that genistein at higher levels decreased hepatic fat accumulation possibly by increasing fatty acid oxidation and uncoupling protein; in low doses, genistein increased mitochondrial enzyme activities in mice with fatty liver and obesity induced by high-fat diets. Taking these data all together could explain why genistein ameliorates liver function in bile duct-ligated rats.

There is a wealth of evidence that HSCs orchestrate most of the important events in liver fibrogenesis. After liver injury, HSCs become activated to a profibrogenic myofibroblast phenotype and can regulate net deposition of collagens and other matrix proteins in the liver. It has been shown that genistein is able to inhibit PDGF-driven proliferative activity of rat HSCs, and also inhibits the TGF-β1-stimulated collagen synthesis. Genistein also influences proliferation of HSCs, suppresses the expression of α-SMA in HSCs, and inhibits the intensity of c-fos, c-jun, and cyclin D1 expression of HSCs. In this work we have shown that genistein reduced the total amount of liver collagen and ameliorated the liver architecture; therefore, it is possible that genistein also influences the HSCs in vivo.

The effects of genistein on other hepatic cells have also been studied. The inhibition of cell proliferation and the induction of apoptosis via activation of caspase-3 have been observed in genistein-treated liver cancer-bearing animals. It has been shown that genistein modulates gene expression in hepatocytes. On the other hand, it has been observed that 100 µmol/L genistein increased the synthesis of nitric oxide by sinusoidal endothelial cells from the early stage (stage I) of fibrosis. Those data suggest that genistein may play an important role in regulating the function of all cells residing in the liver, not only in physiological conditions, but also in liver disease.

Extracellular matrix deposition is a constant feature in liver cirrhosis. Proteolytic enzymes are thought to play a primary role in the degradation of the connective tissue generated in processes such as fibrosis. Different enzymatic systems may contribute to the overall process of ECM degradation, including plasminogen activators, matrix metalloproteinases (i.e., collagensases), and cathepsin D, and it is likely that these enzymatic systems can act either independently or in a concerted manner. The involvement of these powerful proteolytic systems in pathologies such as hepatic fibrosis reinforces the idea that specific mechanisms must exist for its regulation and could be a target for therapeutic strategies in that process. Thus, the increase in the proteolytic activity mediated by genistein administration may be one of the antifibrotic mechanisms of this compound. It has been reported that genistein can modulate the secretion of urokinase type plasminogen activator and other metalloproteinases in human cancer cell lines. Nevertheless, further investi-
gations concerning the modulation of the proteolytic activity in the liver disease by genistein are needed.

In summary, the present report showed that genistein inhibits the fibrosis and cholestasis induced by prolonged biliary obstruction in the rat. Genistein has therapeutic potential against liver fibrosis.

References