

# Estrogen reduces CCL<sub>4</sub>- induced liver fibrosis in rats

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## Abstract

**AIM:** Chronic liver diseases, such as fibrosis or cirrhosis, are more common in men than in women. This gender difference may be related to the effects of sex hormones on the liver. The aim of the present work was to investigate the effects of estrogen on CCL<sub>4</sub>-induced fibrosis of the liver in rats.

**METHODS:** Liver fibrosis was induced in male, female and ovariectomized rats by CCL<sub>4</sub> administration. All the groups were treated with estradiol (1 mg/kg) twice weekly. And tamoxifen was given to male fibrosis model. At the end of 8 weeks, all the rats were killed to study serum indicators and the livers.

**RESULTS:** Estradiol treatment reduced aspartate aminotransferase (AST), alanine aminotransferase (ALT), hyaluronic acid (HA) and type IV collagen (CIV) in sera, suppressed hepatic collagen content, decreased the areas of hepatic stellate cells (HSC) positive for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and lowered the synthesis of hepatic type I collagen significantly in both sexes and ovariectomy fibrotic rats induced by CCL<sub>4</sub> administration. Whereas, tamoxifen had the opposite effect. The fibrotic response of the female liver to CCL<sub>4</sub> treatment was significantly weaker than that of male liver.

**CONCLUSION:** Estradiol reduces CCL<sub>4</sub>-induced hepatic fibrosis in rats. The antifibrogenic role of estrogen in the liver may be one reason for the sex associated differences in the progression from hepatic fibrosis to cirrhosis.

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## INTRODUCTION

Estrogen is frequently used for contraception and treatments of menopausal disorders. Its clinical use has increased steadily during the last years due to reports of decreased morbidity and mortality during postmenopausal estrogen treatment<sup>[1]</sup>. There have been reports of decreased morbidity in cardiovascular

disease, suggesting estrogenic effects on tissues other than on the classic reproductive organs.

Population data have long suggested that chronic liver disease progresses at unequal rates in both sexes for viral hepatitis and other forms of injury with a similar incidence in males and females. In chronic viral hepatitis the major sequelae, such as fibrosis or cirrhosis, are more common in men than in women<sup>[2,3]</sup>. Although establishing the actual rate of fibrosis in a patient would require serial liver biopsy, which is seldom done, a reasonable approximation can be inferred from the incidence of fibrosis-related complications<sup>[4-6]</sup>. The development of cirrhosis is more common in men than in women (2.3 to 2.6:1). Although the liver is not a classic sex hormone target, livers in both men and women have been shown to contain estrogen receptors and respond to estrogens by regulating liver function. Therefore, sex hormones may play a role in the progression from hepatic fibrosis to cirrhosis. It showed that estradiol treatment resulted in reducing hepatic fibrosis in rats induced by dimethylnitrosamine (DMN). However, much current evidence suggests that women develop alcoholic liver disease at lower levels of alcohol intake and over a shorter period of time as compared to men. In other words, females are more susceptible to alcohol-induced liver injury than males<sup>[7]</sup>. The specific mechanisms concerning a gender-related difference in susceptibility are largely unknown.

CCL<sub>4</sub>-induced fibrosis shares several characteristics with human fibrosis of different etiologies; thus, it is an adequate model of human fibrosis<sup>[8-10]</sup>. The aim of the present work was to study the effects of estrogen on CCL<sub>4</sub>-induced fibrosis of the liver in rats, and to investigate the possible mechanisms.

## MATERIALS AND METHODS

### Animals

Male and female Sprague-Dawley rats (Experimental Animal Holding Unit of Shaanxi Province, China) were housed in a temperature-humidity-controlled environment with 12-h light-dark cycles (lights on from 07:00 to 19:00) and had unrestricted access to food and water. Forty male rats, weighing 220±21 g, corresponding to an age of approximately 10 weeks, were divided into four groups of ten each. For CCL<sub>4</sub> group, 400 mL/L CCL<sub>4</sub> in peanut oil were injected subcutaneously at a dose of 2 mL/kg twice weekly, and the first dosage was doubled. The estrogen group, apart from the used of CCL<sub>4</sub>, was treated subcutaneously with estradiol 1 mg/kg twice weekly (The Ninth Pharmaceutical Plant of Shanghai, China). The anti-estrogen group, along with the CCL<sub>4</sub> treatment described above, was given Tamoxifen 6 mg/kg every day orally (The First Pharmaceutical Plant of Suzhou, China). The rats were fed a modified high fat diet containing 5 g/kg cholesterol and 200 g/kg pig oil. The control group was given normal food and water, and received injection of peanut oil vehicle twice weekly.

Fifty female rats 10 weeks old, weighing 208±17 g, were divided into five groups with ten each. The ovariectomy (Ovx) group was initiated with a bilateral ovariectomy and the sham operation group was initiated with just a sham operation. The

two estrogen groups, with bilateral ovariectomy and with sham operation, were treated subcutaneously with estradiol (1 mg/kg twice weekly). All of the above four groups received 400 ml/L of CCL<sub>4</sub> in peanut oil at a dose of 2 ml/kg twice weekly, and were fed with a high fat diet containing 5 g/kg cholesterol and 200 g/kg pig oil. The CCL<sub>4</sub> and estradiol were used after 2 weeks of operation. The control group was given normal food and water, and received injection of peanut oil vehicle twice weekly.

At the end of the 8-week experimental period, all the rats were fasted overnight and put to death by cervical dislocation after anaesthetised by intramuscular injection of sodium pentobarbital (40 mg/kg). Blood was collected from the animals and the serum obtained was analysed. The liver was removed rapidly.

#### Estimation of serum indicators

In serum, activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by a 917-Hitachi Automatic Analyzer. Serum hyaluronic acid (HA) and type IV collagen (CIV) concentrations were measured radioimmunologically using commercial kit (Shanghai Navy Medical Institute, China).

#### Parameters of hepatic antioxidation

Parameters of antioxidation in the liver was determined by measuring the levels of hepatic malondialdehyde (MDA) and superoxide dismutase (SOD) (kit: Jiancheng Medical Institute, Nanjing, China).

#### Histopathological study

Excised liver tissues from each rat were fixed in 100 ml/L neutral formalin, embedded in paraffin, and stained with hematoxylin-eosin (HE) and masson's trichrome. The evaluation of hepatic fibrosis was determined by a semi-quantitative method to assess the degree of histologic injury in chronic hepatic fibrosis<sup>[11,12]</sup>.

#### Immunohistochemical examination

Liver tissue sections were mounted on slides, deparaffinized in xylene, and rehydrated in alcohol. The level of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Neomarkers, USA), type I collagens (Boster, Wuhan, China), transforming growth factor  $\beta_1$  (TGF  $\beta_1$ ) and platelet-derived growth factor (PDGF) (Dako, USA) were determined by immunohistochemical methods in female

groups. Based on the extent of histological staging, the  $\alpha$ -SMA, type I collagens, TGF $\beta_1$  and PDGF positive cells were expressed as a percentage of the total area of the specimen.

#### Statistical analysis

Data are presented as  $\bar{x} \pm s$  unless otherwise indicated. The Mann-Whitney *u* test for nonparametric and unpaired values, student's *t*-test or Fisher's exact test was used as appropriate. Results were considered significant when  $P < 0.05$ .

## RESULTS

#### Changes of serum indicators and hepatic antioxidation data

At the end of 8-week experimental period, 8 rats were dead because of infection at the region of injection and hepatic crack by unsuitable handling. Table 1 gives the values for the activities of the serum indicator enzymes, the markers of hepatic fibrosis, and the hepatic antioxidation data.

It is evident that CCL<sub>4</sub> produced a marked increase in the activities of serum ALT and AST in both male and female rats. Although the extent of that was lower in female group than in male group, it was not statistically significant ( $P > 0.05$ ). The CCL<sub>4</sub> plus estradiol group showed a significant decrease in the enzyme levels, but the levels were still higher than those of control groups. In ovariectomy rats, when CCL<sub>4</sub> were given, the enzyme levels were higher than those of the sham operation rats in both estradiol used or none-used groups, but the differences were not statistically significant ( $P > 0.05$ ). The levels of serum ALT and AST in Tamoxifen group were significantly higher than those of the CCL<sub>4</sub> group and estrogen used groups.

As for the changing trend of fibrotic markers in serums, HA and CIV were similar with those of the enzyme levels in all groups. The results showed that the levels of HA and CIV in CCL<sub>4</sub> used groups were significantly higher than those of control groups, especially in male group. Tamoxifen could increase the extent of that and estrogen could decrease it significantly. In ovariectomy groups, the HA and CIV were significantly higher than those of sham operation groups.

Tissue antioxidation data indicated that MDA was increased and SOD was decreased in CCL<sub>4</sub> treated rats significantly, whereas estradiol had the opposite effect. The MDA was higher and SOD was lower in males than those of females treated with CCL<sub>4</sub>.

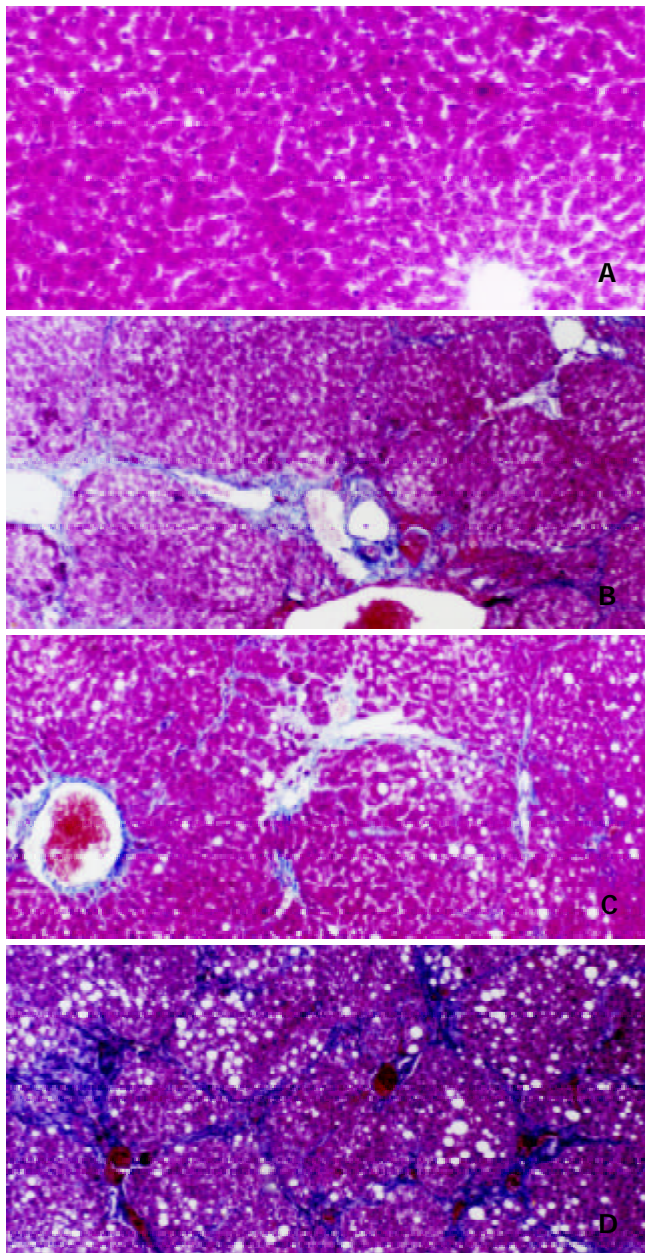
**Table 1** Liver enzymes, serum fibrosis indicators and hepatic antioxidation ( $\bar{x} \pm s$ )

Group	<i>n</i>	ALT (nkat/L)	AST (nkat/L)	HA ( $\mu$ g/L)	CIV ( $\mu$ g/L)	MDA (nmol/g)	SOD (kNU/g)
<b>Males</b>							
Control	10	31 $\pm$ 6	66 $\pm$ 18	115 $\pm$ 31	18 $\pm$ 5	3.1 $\pm$ 0.9	8.0 $\pm$ 1.6
CCL <sub>4</sub>	10	576 $\pm$ 262 <sup>a</sup>	699 $\pm$ 241 <sup>a</sup>	530 $\pm$ 122 <sup>a</sup>	54 $\pm$ 14 <sup>a</sup>	10.5 $\pm$ 3.4 <sup>a</sup>	2.2 $\pm$ 1.1 <sup>a</sup>
CCL <sub>4</sub> +E	8	355 $\pm$ 125 <sup>c</sup>	314 $\pm$ 179 <sup>c</sup>	232 $\pm$ 78 <sup>c</sup>	30 $\pm$ 10 <sup>c</sup>	6.6 $\pm$ 2.8 <sup>c</sup>	5.0 $\pm$ 1.6 <sup>c</sup>
CCL <sub>4</sub> +Tam	8	884 $\pm$ 294 <sup>c</sup>	1073 $\pm$ 453 <sup>c</sup>	703 $\pm$ 187 <sup>c</sup>	69 $\pm$ 15 <sup>c</sup>		
<b>Females</b>							
Control	10	35.8 $\pm$ 7.9	64.5 $\pm$ 20.8	121 $\pm$ 26	17 $\pm$ 4	3.0 $\pm$ 0.8	8.7 $\pm$ 2.8
CCL <sub>4</sub>	9	540 $\pm$ 252 <sup>a</sup>	631 $\pm$ 268 <sup>a</sup>	388 $\pm$ 81 <sup>a</sup>	41 $\pm$ 11 <sup>a</sup>	7.1 $\pm$ 2.1 <sup>a</sup>	4.0 $\pm$ 1.5 <sup>a</sup>
CCL <sub>4</sub> +Ovx	10	658 $\pm$ 220	697 $\pm$ 240	586 $\pm$ 145 <sup>c</sup>	53 $\pm$ 14 <sup>c</sup>	9.1 $\pm$ 2.9 <sup>c</sup>	2.8 $\pm$ 1.0 <sup>c</sup>
CCL <sub>4</sub> +E	8	314 $\pm$ 163 <sup>c</sup>	302 $\pm$ 153 <sup>c</sup>	267 $\pm$ 83 <sup>c</sup>	29 $\pm$ 7 <sup>c</sup>	4.7 $\pm$ 2.2 <sup>c</sup>	5.9 $\pm$ 2.0 <sup>c</sup>
CCL <sub>4</sub> +Ovx+E	9	311 $\pm$ 146 <sup>c</sup>	321 $\pm$ 121 <sup>c</sup>	236 $\pm$ 119 <sup>c</sup>	31 $\pm$ 7 <sup>c</sup>	4.8 $\pm$ 2.3 <sup>c</sup>	6.2 $\pm$ 2.5 <sup>c</sup>

<sup>a</sup> $P < 0.05$ , vs control; <sup>c</sup> $P < 0.05$ , vs CCL<sub>4</sub>

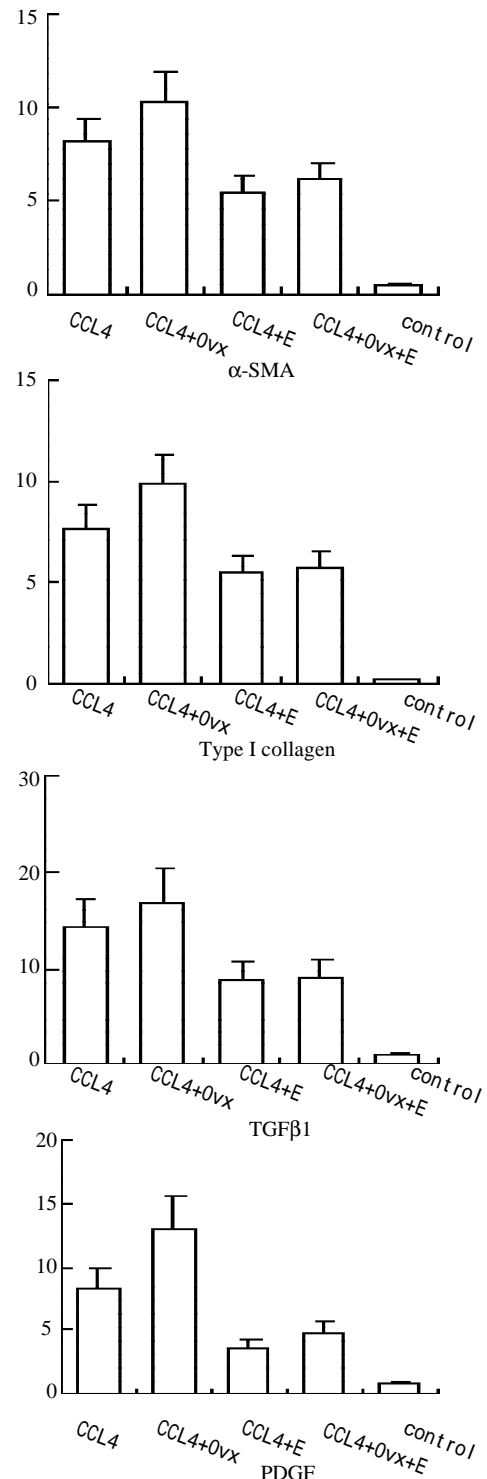
**Histopathological and immunohistochemical changes**

The control livers showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Prolonged administration of CCL<sub>4</sub> causes severe pathological damages: inflammation, necrosis, and collagen deposition (Figure 1B,male). The semiquantitative hepatic collagen staging value was 3.3±0.7 in males, and 2.4±1.1 in females. It was showed that the staging value was significantly decreased in female rats. After administration of estradiol, the extent of hepatic fibrosis was significantly weaker than that of CCL<sub>4</sub> groups (Figure 1C:male): the semiquantitative staging value was 2.0±1.1 in males and 1.6±0.9 in females respectively. Ovariectomy significantly increased the staging value (3.1±0.7). Moreover, the staging value was highest when given Tamoxifen to the experimented rats (3.7±0.5 Figure 1D).



**Figure 1** Effects of estradiol and tamoxifen on the histology of CCL<sub>4</sub> induced fibrotic rat liver. Masson trichome stain, scale bar=40 μ m, original magnification, ×100  
 1A: Normal rat liver; 1B: CCL<sub>4</sub> group shows fibrosis; 1C: Estrogen group with less fibrosis than in group B; 1D: Tamoxifen group shows marked fibrosis than in group B.

Analysis of α-SMA, an activation marker of rat hepatic stellate cells (HSC) by immunohistochemistry showed staining in vascular smooth muscle cell of control rat livers, but not in sinusoids. In the CCL<sub>4</sub> model, the positive cells of α-SMA, type I collagen, TGFβ<sub>1</sub> and PDGF within centrilobular and periportal fibrotic bands. The percentage areas of these staining in the liver of female rats were showed in Figure 2. The results suggest that administration of CCL<sub>4</sub> significantly increased the percentage areas of all of the four marker staining. Ovariectomy group has a marked higher percentage area than that of CCL<sub>4</sub> group and it could be significantly suppressed by estradiol used.



**Figure 2** Percentage area (%) of α-SMA, type I collagen, TGFβ<sub>1</sub>, and PDGF in female rats

## DISCUSSION

Hepatic fibrosis is usually initiated by hepatocyte damage, leading to recruitment of inflammatory cells and platelets, activation of kupffer cells and subsequent release of cytokines and growth factors (e.g. TGF $\beta_1$  and PDGF)<sup>[13,14]</sup>. These factors probably link the inflammatory and reparative phase of liver cirrhosis, by activating HSC<sup>[15-18]</sup>. Upon activation, HSC proliferate and transform into myofibroblast-like cells that deposit large amounts of connective tissue components<sup>[19-27]</sup>.

The present study showed that estradiol reduces CCL<sub>4</sub>-induced hepatic fibrosis in rats. Estradiol administration reduces HA and CIV in serums, suppresses hepatic collagen content, reduces the areas of HSC positive for  $\alpha$ -SMA, and lowers the synthesis of hepatic type I collagen in both sexes. The fibrotic response of the female liver to CCL<sub>4</sub> treatment was significantly weaker than that of male liver. It suggested that physiological levels of estrogen have an antifibrogenic effect. These effects of estrogen were also confirmed by ovariectomy in female rats at the time of CCL<sub>4</sub> administration. These findings suggest that the antifibrogenic role of estrogen in the liver may be one reason for the sex associated differences in the progression from hepatic fibrosis to cirrhosis.

Hepatic fibrogenesis is often associated with hepatocellular necrosis and inflammation accompanied by the repair processes<sup>[28-29]</sup>. Chronic administration of CCL<sub>4</sub> caused fibrosis as indicated by an increase in serum marker enzymes. Raised serum enzyme levels in CCL<sub>4</sub>-injected rats can be attributed to the damaged hepatocellular structural integrity<sup>[30-32]</sup>. The administration of estradiol in this study seems to decrease the serum enzymes (ALT, AST), and then preserve the structural integrity of the hepatocellular membrane. Decreased hepatocyte damage suppressing the stimulant effect to the Kupffer cells and subsequent by lower the HSC activation<sup>[33]</sup>.

Peroxidation of lipids can dramatically change the properties of biological membranes, resulting in severe cell damage and could play a significant role in the pathogenesis of disease. It has showed that lipid peroxidation, free-radical-mediated process, and certain lipid peroxidation products induce genetic overexpression of fibrogenic cytokines and increase the synthesis of collagen. Free radicals and MDA can stimulate the synthesis of collagen and initiate the activation of HSC<sup>[34,35]</sup>. Relevant to the latter findings, estradiol and its derivatives are strong endogenous anti-oxidants that reduce lipid peroxide levels in liver and serum. Free radicals, generated mainly by Kupffer cells, are thought to cause tissue injury by initiating lipid peroxidation and irreversibly modifying membrane structure. The present study shows that CCL<sub>4</sub> administration leads to parallel increase in MDA and collagen, and subsequent decrease in SOD, a radical scavenging agent, and that estradiol had the opposite effect. These findings suggest that the antifibrotic effect of estradiol may be caused, at least in part, by its radical scavenging action or antioxidant activity.

Cell proliferation,  $\alpha$ -SMA expression, retinoid disappearance, and the formation of collagens and other ECM materials are characteristics of the activated phenotype of HSC<sup>[36]</sup>. So HSC are regarded as the primary target cells of hepatic fibrogenesis. Studies in vitro have shown that HSC and their activated counterparts may be induced to proliferate by polypeptide growth factors and cytokines, such as TGF $\beta_1$  and PDGF<sup>[37,38]</sup>. As these growth factors are produced by infiltrating inflammatory cells, Kupffer cells and sinusoidal endothelial cells, they might act as paracrine mediators which trigger the transformation of HSC *in vivo*. The present data show that estradiol suppresses HSC proliferation and parallel with inhibit TGF $\beta_1$  and PDGF expression in CCL<sub>4</sub>-induced

fibrosis in male and female rats. These findings suggest that estrogen may exert its suppressive effect on hepatic fibrosis by indirectly modulating the synthesis and releasing of cytokines and other growth factors which in turn altering HSC activation and proliferation.

Chronic fibrotic diseases can differ from each other in etiology. But, in terms of pathogenesis, they share some basic common features<sup>[1]</sup>. For instance, three serious chronic disease-atherosclerosis, glomerulosclerosis, and liver fibrosis-may appear very different in their development. In all three, though, a central, and indeed an essential, role is played by macrophages and by ECM-producing cells: smooth muscle cells (SMC) in atherosclerosis, mesangial cell in glomerulosclerosis, and HSC in liver fibrosis. The three types of cells have many properties in common both structurally and functionally, including a changes in cell biology with deposition of matrix proteins. Therefore, factors which affect the development of atherosclerosis or glomerulosclerosis may affect liver fibrosis by similar mechanisms. Studies show that estradiol suppresses atherosclerosis and glomerulosclerosis in rats by directly affecting the estrogen receptor on SMC and mesangial cell. Livers in both male and Female rats have shown to contain high affinity, low capacity estrogen receptors and respond to estrogen by regulating liver function. In the present study, Tamoxifen, an antiestrogen act by occupying the estrogen-binding site of the receptor protein, increases fibrogenesis in CCL<sub>4</sub>-induced fibrosis of the liver. It suggests that estradiol may suppress hepatic fibrosis also by a direct receptor mechanism. This remains to be confirmed.

In conclusion, estrogen may play an important role as an endogenous fibrosuppressant, accounting for sex-associated differences in the progression from hepatic fibrosis to cirrhosis. The following mechanisms have been hypothesized to explain the antifibrogenic effect of estrogens: (A) a hepatocellular membrane protection and radical scavenging action. (B) a modulation of HSC proliferation and collagen synthesis, (C) a modulation in the expression of pro-and anti-fibrogenic cytokines and may be (D) a estrogen receptor mechanism. However, the real importance of these mechanisms is still to be elucidated.

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