Estrogen reduces CCL₄-induced liver fibrosis in rats

Jun-Wang Xu, Jun Gong, Xin-Ming Chang, Jin-Yan Luo, Lei Dong, Zhi-Ming Hao, Ai Jia, Gui-Ping Xu

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 CCL₄-induced fibrosis of the liver in rats.

METHODS: Liver fibrosis was induced in male, female and ovariectomized rats by CCL₄ administration. All the groups were treated with estradiol (1 mg/kg) twice weekly. 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 α-SMA, and lowered the synthesis of hepatic type I collagen significantly in both sexes and ovariectomy fibrotic rats induced by CCL₄ administration. Whereas, tamoxifen had the opposite effect. The fibrotic response of the female liver to CCL₄ treatment was significantly weaker than that of male liver.

CONCLUSION: Estradiol reduces CCL₄-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.


INTRODUCTION

Estrogen is frequently used for anticonception 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.[1] 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.[2,3] 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[4,6]. 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.[7] The specific mechanisms concerning a gender-related difference in susceptibility are largely unknown.

CCL₄-induced fibrosis shares several characteristics with human fibrosis of different etiologies; thus, it is an adequate model of human fibrosis[8,9]. The aim of the present work was to study the effects of estrogen on CCL₄-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 CCL₄ group, 400 mL/L CCL₄ 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 use of CCL₄, was treated subcutaneously with estradiol 1 mg/kg twice weekly (The Ninth Pharmaceutical Plant of Shanghai, China). The anti-estrogen group, along with the CCL₄ 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 CCL4 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 CCL4 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[11,12].

Immunohistochemical examination
Liver tissue sections were mounted on slides, deparaffinized in xylene, and rehydrated in alcohol. The level of α-smooth muscle actin (α-SMA) (Neomarkers, USA), type I collagens (Boster, Wuhan, China), transforming growth factor β1 (TGF β1) and platelet-derived growth factor (PDGF) (Dako, USA) were determined by immunohistochemical methods in female groups. Based on the extent of histological staging, the α-SMA, type I collagens, TGFβ1 and PDGF positive cells were expressed as a percentage of the total area of the specimen.

Statistical analysis
Data are presented as x±s vs 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 CCL4 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 CCL4 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 CCL4 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 CCL4 group and estrogen used groups.

As for the changing trend of fibrotic markers in sera, 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 CCL4 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.

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**Histopathological and immunohistochemical changes**

The control livers showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Prolonged administration of CCL₄ 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 CCL₄ 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).

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 CCL₄ model, the positive cells of α-SMA, type I collagen, TGFβ₁, 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 CCL₄ significantly increased the percentage areas of all of the four marker staining. Ovariectomy group has a marked higher percentage area than that of CCL₄ group and it could be significantly suppressed by estradiol used.

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**Figure 1** Effects of estradiol and tamoxifen on the histology of CCL₄-induced fibrotic rat liver. Masson trichome stain, scale bar=40μm, original magnification, ×100

1A: Normal rat liver; 1B: CCL₄ group shows fibrosis; 1C: Estrogen group with less fibrosis than in group B; 1D: Tamoxifen group shows marked fibrosis than in group B.

**Figure 2** Percentage area (%) of α-SMA, type I collagen, TGFβ₁, 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. TGFβ and PDGF)[13,14]. These factors probably link the inflammatory and reparative phase of liver cirrhosis, by activating HSC[15-18]. Upon activation, HSC proliferate and transform into myofibroblast-like cells that deposit large amounts of connective tissue components[19-27].

The present study showed that estradiol reduces CCL4-induced hepatic fibrosis in rats. Estradiol administration reduces HA and CIV in sera, suppresses hepatic collagen content, reduces the areas of HSC positive for α-SMA, and lowers the synthesis of hepatic type I collagen in both sexes. The fibrotic response of the female liver to CCL4 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 CCL4 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[28-29]. Chronic administration of CCL4 caused fibrosis as indicated by an increase in serum marker enzymes. Raised serum enzyme levels in CCL4-injected rats can be attributed to the damaged hepatocellular structural integrity[30-32]. 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[33].

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 induce genetic overexpression of fibrogenic cytokines and damage and could play a significant role in the pathogenesis of type IV collagen in the progression from hepatic fibrosis to cirrhosis.

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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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