Antifibrotic Effect of Spironolactone in Rats Induced by CCl_4 is Mediated by Down–Regulation of TGF–β1 and PDGF–BB and HSC

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Abstract **Objective** To assess the effect of spironolactone in the prevention of hepatic fibrosis in rats and to explore its antifibrotic mechanism. **Methods** 90 SD rats were randomly allocated into three groups. Group A (normal controls) of 8 rats; Group B (fibrotic model controls) of 42 rats; Group C (Spironolactone prevention) of 40 rats. The rats of group A, fed by normal food, were injected with peanut oil subcutaneously. The rats of group B were given compound factors to induce liver fibrosis. Spironolactone preventing group were given 100mg/kg spironolactone, besides, the methods of making models were the same as those of the model group. At the end of 8 week, all the rats were sacrificed. Blood was taken from eyes and the level of HA, LN, PC Ⅲ and CIV were examined. Liver tissues were stained with HE and VG. Tissue morphological change was examined with microscopy. The degree of hepatic fibrosis was assessed by an image analyze system. Moreover, the expression of TGF–β1, PDGF–BB and α–SMA in hepatic tissue were detected with immunohistochemical methods. **Results** The levels of HA, LN, PC Ⅲ and CIV in spironolactone group were greatly less than those in model group(P<0.05). Histological observation indicated that the grade of fibrosis and the area of collagen in rats of the model group were significantly higher than those of the spironolactone group(P<0.05); The expression of TGF–β1, PDGF–BB and α–smooth muscle actin in hepatic tissue in the group of spironolactone were significantly less than those in the model group(P<0.05). **Conclusion** Spironolactone can obviously inhibit the formation of liver fibrosis in rats through decreasing the secretion of TGF–β1,PDGF–BB and inhibiting the activation of hepatic stellate cell.

Key words Spironolactone; Hepatic fibrosis; Hepatic stellate cell; α–smooth muscle actin; TGF–β1; PDGF–BB

Hepatic fibrosis is the result of the disequilibrium between synthesis and degradation of extracellular matrix (ECM) components, which leads to significant morbidity and mortality. The accumulation of components of the ECM is the main pathologic feature of hepatic fibrosis. Numerous pharmaceutical agents have been tried with varying degrees of success, but with unacceptable side effects in long–term therapy. Spironolactone(SPN) is a frequently used diuretic in patients with cirrhosis. In recent years, SPN has been reported to prevent extracellular matrix accumulation in myocardial, vascular and renal fibrosis [1~3]. Especially, SPN has been tested with promising results in patients with myocardial fibrosis[4]. The aim of the study was to study the preventive effects of SPN in rat liver fibrosis induced by CCl4 and to explore the possible mechanism.

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MATERIALS AND METHODS

Materials

Male Sprague–Dawley (SD) rats weighing 230±28g were purchased from animal center of Xi´an Jiaotong University. SPN was produced by Huanghai Pharmaceutical Company of Jiangsu. HA, LN, PC Ⅲ and CⅣ were purchased from Navy Medicine Research Institute of Shanghai. TGF–β1, PDGF–BB and α–SMA monoclonal antibody were purchased from Maxim Biotechnology Company.

Methods

90 male SD rats used for the study were housed in conventional cages with free access to water and rodent chow at 20−22℃ with a 12−hour light−dark cycle. All procedures involving the use of laboratory animals were in accordance with National Institutes of Health guidelines. Animals were randomly divided into three groups: group A(normal controls, n=8), group B (fibrotic model controls, n=42), group C (SPN preventing group, n=40), in addition to CCl_4, were given SPN (100 mg/kg body weight) gavage per day. Experimental liver fibrosis was produced using an established protocol [5] that involved ① an initial dose of 5.0ml CCl_4/kg, sc, using 40% CCl_4; ② injection of 3.0ml/kg, sc, of 40% CCl_4 in peanut oil (1.2ml CCl_4/kg), twice a week; ③ high fat diet (20% fat, 0.5% cholesterol); and ④30% alcohol in drinking water every other day. After 8 weeks of treatment, liver fibrosis was established. In rats of group C, SPN was given(100mg/kg body weight) gavage per day. The dose and duration of SPN treatment were based on the pilot experiments and previous studies[5]. The rats in normal control group and CCl_4 protocol group received saline gavage instead of SPN. All the animals received humane care and our study complies with the institution´s guidelines. At the end of 8th week, all animals were sacrificed.

Serum studies

Blood samples from eye balls were immediately centrifuged at 4℃ and plasma was kept at −20℃ until the assays were performed. HA, LN, PC Ⅲ and CⅣ were determined using a radioimmunoassay technique.

Histopathological examinations and image analysis of liver fibrosis

Liver samples from all animals were processed for light microscope. Tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin, cut in 5μm pieces and mounted on the slide. Paraffin sections were stained with hematoxylin and eosin and Van Gieson. For morphometric studies, three liver fragments were randomly taken in the right, median and left liver lobes of each rat. Liver fragments were fixed in 10% solution of formaldehyde in 0.1mol/L phosphate−buffered saline and embedded in paraffin. Collagen expression was detected with standard Van Gieson staining. Collagen surface density was measured with a colorful medical image analysis system (CMIAS). Collagen surface density was expressed as the percent of collagen per analyzed parenchymal surface. Relative total liver fibrosis was expressed as the mean fibrosis percentage in the three liver fragments and was called area of fibrosis.

The expression of TGF−β1,PDGF−BB and α−SMA

Immunohistochemical method was used to detect the expression of TGF−β1, PDGF−BB and α−SMA. The CMIAS was also used to evaluate the expression of TGF−β1, PDGF−BB and α−SMA. The methods were the same as those for image analysis of liver fibrosis as described above.

Statistics analysis

All results were expressed as the means±standard deviation ($\bar{x} \pm s$), and statistical analysis was carried out with SPSS, statistical significance was considered when $P<0.05$.

RESULTS

General characteristics of rats

Liver and body weight: Initial body weight was not significantly different between the groups in each model, the decrease in body weight was significantly different between the model group and the control group, the liver weight was significantly increased in model rats ($P<0.05$) compared to control group rats. Liver weight was not significantly modified in the SPN group compared with the placebo group in both models.

HA, LN, PC Ⅲ, CⅣ (μg/L) and Collagen density (see table 1)

SPN inhibit the secretion of TGF−β1, PDGF−BB and activation of HSC(see table2)

Histopathological change

Tissue specimen: The liver tissue was dark and red and the surface of liver was full of symmetrical and crisp

Table 1 Fibrosis data

Group	Number	HA	PCⅢ	LN	CⅣ	Collagen density
A	8	205.37±68.58	27.85±4.51	40.10±4.07	8.57±1.47	0.69±0.20
B	42	615.15±80.48	46.42±3.84	96.86±8.44	18.73±2.85	9.47±0.87[a]
C	40	536.59±59.95	41.38±3.34	86.53±8.43	15.84±2.24	7.16±0.73[ab]

Collagen density=collagen area/liver tissue area in field of vision

a. P <0.01 vs normal control group; b. P<0.05 vs model group

Table 2 The expression of TGF-β1、PDGF-BB and α-SMA in liver tissue (μg/L)

Group	Number	TGF-β1	PDGF-BB	α-SMA
A	8	0.72±0.26	0.67±0.14	0.63±0.23
B	42	13.47±3.13[a]	11.37±2.30[a]	11.21±0.97[a]
C	40	9.28±2.67[ab]	8.94±2.04[ab]	8.45±0.86[ab]

a. P<0.01 vs normal control group; b. P<0.05 vs model group

grana in model group. In SPN group, the color, texture, magnitude and surface of liver tissue were all improved obviously compared with the model group.

Light scope: The hepatocytes of the control group showed normal architectural pattern. In model group, the hepatocytes were denaturalized generally and presented different degrees of necrosis(Fig.1a). VG staining showed a great deal of collagen fibers deposited at portal areas and were connected to each other. The structure of lobules was destroyed and pseudo lobules had formed (Fig.2a). There was a significant increase in the area of fibrosis in model rats compared with the control rats, 67% of the specimens belong to grade3. Liver sections of SPN therapeutic group showed reduction of liver necrosis and inflammation, congestion of the central veins was less pronounced, sinusoidal widening less prominent (Fig.1b), hepatic fibrosis less obviously(Fig. 2b). SPN significantly decreased the area of fibrosis which belonged to grade2.

The expression of TGF-β1, PDGF-BB and α-SMA in different groups

The positive signals of TGF-β1, PDGF-BB and α-SMA were stained brown. At the end of the 8th week, TGF-β1 positive signals were situated in the sinusoidal wall, perisinusoidal, necrosis zone and portal area(Fig.3a); and PDGF-BB positive signal were stained brown situated at portal area and fiber interval in model group (Fig.4a). In normal control group, α-SMA positive cell wasn´t seen. At the end of the 8th week, a mass of α-SMA positive cells can be seen in the dilated portal area and fiber interval in model group (Fig.5a). With the development of hepatic fibrosis, the expression of TGF-β1, PDGF-BB and α-SMA in liver were gradually obvious. Compared with the model group, the expression of TGF-β1,PDGF-BB and α-SMA in liver in SPN therapeutic group were significantly lower (P<0.01) (Fig.3b, 4b, 5b)

DISCUSSION

In the long term, it´s generally suggested that aldosterone is a synthesis in adrenal glands only and an action exclusively in epithelial tissues. Human tissue cultures and animal models have established that aldosterone synthase ($CYPIIB_2$), the enzyme responsible for the last step in aldosterone biosynthesis, has been identified in the heart, blood vessels, and liver, which suggests that aldosterone also may be produced in these tissues[6,7]. In vitro study, $CYPIIB_2$ mRNA was expressed in hepatic stellate cell [8].

Until recently, SPN was considered only as an antagonist at the aldosterone receptors of the epithelial cells of the kidney and was used clinically in the treatment of hyperaldosteronism and as a k (+)-sparing di-

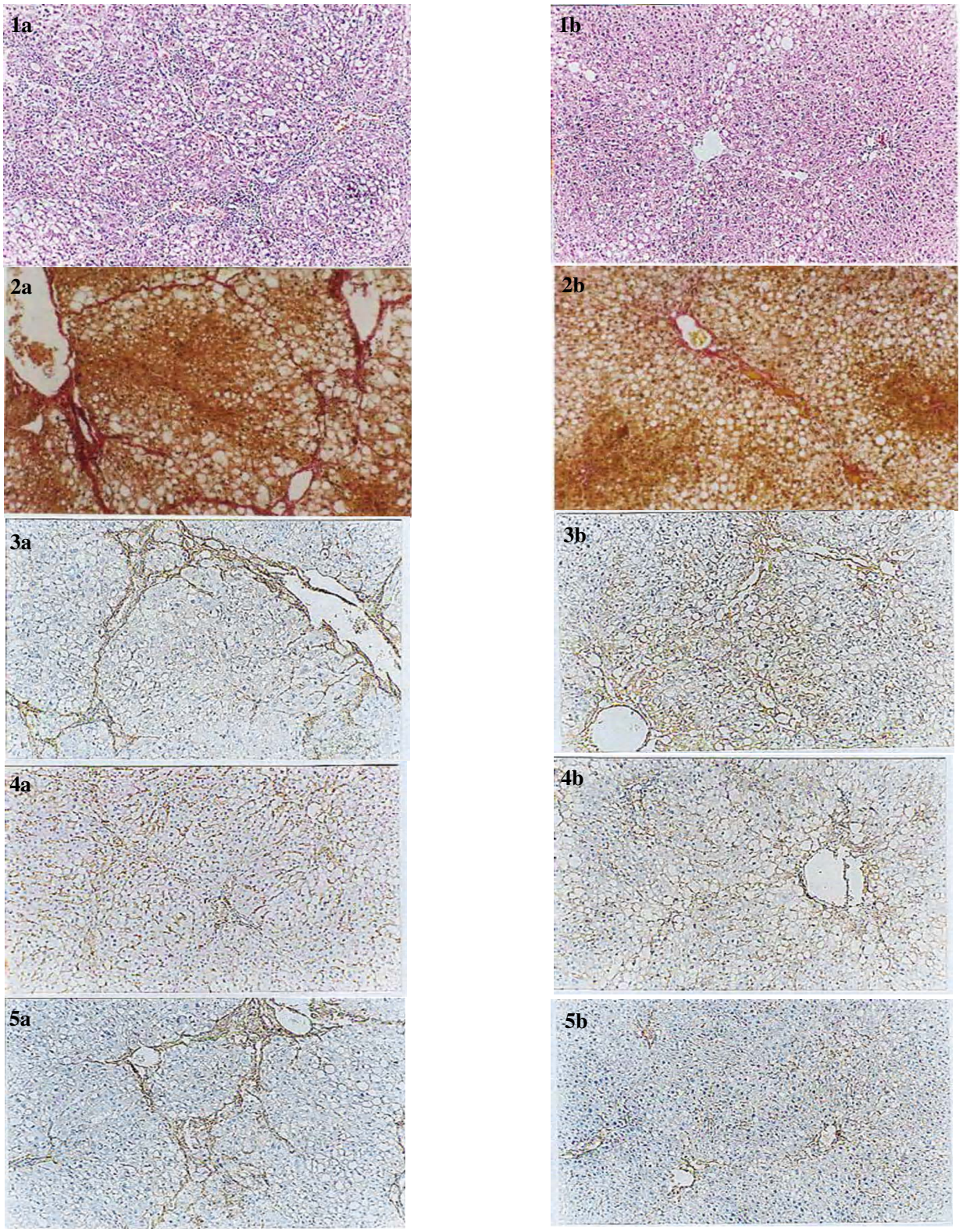

Fig. 1 HE staining of model group (a) and SPN group (b) (HE×100)

Fig. 2 VG staining of model group (a) and SPN group (b) (VG×100)

Fig. 3 Immunohistochemitry of TGF−β1 in model group (a) and SPN group (b) (SP×100)

Fig. 4 Immunohistochemitry of PDGF−BBin the model group (a) and SPN group (b) at the end of 8th week (SP×100)

Fig. 5 Immunohistochemitry of α−SMA in the model group (a) and SPN group (b) at the end of 8th week (SP×100)

uretic. Spironolactone reversed aldosterone −induced cardiac fibrosis by a cardiac action. It also reduces vascular fibrosis and inhibits angiogenesis [9]. In addition, SPN ameliorates rat pulmonary fibrosis induced by bleomycin A_5 [10]. In the present study we have found that the generally characteristics of rats in SPN group were better than those in model group. Spironolactone significantly reduced HA, PCIII, LN and CⅣ levels, and collagen in liver tissue. Histological examination also showed that spironolactone evidently alleviated the progression of hepatic fibrosis. Under the light scope, denaturation, necrosis of hepatocytes and infiltration of inflammation cell in SPN group were also less severe than those in model group. In model group, we can see masses of collagen were deposited in fiber spatial and connected to form pseudolobule. Treatment with the SPN resulted in decreased periportal and bridging necrosis, intralobular degeneration, and lobular and periportal inflammation, with a greater improvement in SPN group than in model group. All results indicated that spironolactone could inhibit the progression of hepatic fibrosis induced by CCl_4 in rats, as previously described [11, 12].

To address the way in which SPN results in a significant reduction in fibrosis, we have investigated the effect of treatment with SPN on the expression of α−SMA, TGF−β1 and PDGF−BB. α−SMA is the symbol of activation of the HSC. During hepatic fibrogenesis, activated HSC are the major source of extracellular matrix constituents. After HSC activation, overall collagen levels increase greatly, with type I collagen as the predominant form [13]. In the present study, we can see the expression of α−SMA in SPN group was greatly lower than that in model group. The activation of HSC is also accompanied by elevated collagen secretion.

Numerous experimental and human studies provide evidence for TGF−β1 as a key mediator of tissue fibrosis, because this cytokine promotes not only the activation and transdifferentiation of retinoid−storing hepatic stellate cells to extracellular matrix producing myofibroblasts but also stimulates the expression of a broad spectrum of extracellular matrix molecules and inhibits simultaneously their degradation by downregulation of matrix metalloproteinases and upregulation of their respective inhibitors [14]. PDGF is a well recognized mitogen for HSC in vitro [15], and an important fibrogenic factor in chronic human liver disease and toxic liver injury in rats. Our findings showed that the expressions of TGF−β1 and PDGF−BB in spironolactone groups were greatly less than those in fibrotic groups. But we don´t know exactly whether this was the result of direct effect of spironolactone or indirect effect of spironolactone through inhibiting activation of a−SMA. During hepatic fibrogenesis, HSCs in the necrotic area are probably activated by locally excreted cytokines and/or conformational changes demonstrated are TGF−β1 [16] and platelet derived growth factor [17]. TGF−β1 and PDGF−BB increases the production of the extracellular matrices of HSCs and also stimulates autocrine TGF−β1 and PDGF−BB excretion[18].

Whether TGF−β1 and PDGF−BB are down−regulated by a direct action of SPN, or SPN initially supressed HSC and then the cytokines were produced secondarily, and both mechanisms proceed simultaneously, were not resolved in this study. Further study is necessary for better understanding of these problems.

In conclusion, the administration of SPN was beneficial in treating hepatic fibrosis induced by CCl_4. Furthermore, the present study also suggests that the inhibitory effect of SPN on hepatic fibrosis is associated with its ability to inhibit the production of TGF−β1 and PDGF−BB by activated HSC.

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