

## Effect of Sho-saiko-to Extract on Hepatic Inflammation and Fibrosis in Dimethylnitrosamine Induced Liver Injury Rats

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Received May 13, 2002; accepted August 8, 2002; published online August 20, 2002

Sho-saiko-to extract, a Chinese herbal medicine, is widely used for treatment of chronic hepatitis in Japan. However, it is not clear what conditions Sho-saiko-to extract improves hepatic inflammation and fibrosis. We therefore induced various stages of liver injury in model rats and administered Sho-saiko-to extract. We then evaluated the liver inflammation and liver fibrosis-improving effects of Sho-saiko-to extract. The liver injury model rats were produced by administration of various doses of dimethylnitrosamine (DMN) and Sho-saiko-to extract was administered to these rats. Then the liver inflammation and fibrosis-improving effects of Sho-saiko-to extract were evaluated according to L-aspartate aminotransferase (AST), L-alanine aminotransferase (ALT), liver retinoid levels, levels of hydroxyproline, Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), and the liver fibrosis area. These indicators depended on the total doses of DMN. The ability of Sho-saiko-to extract to improve liver inflammation and fibrosis was limited to the following levels of the respective parameters: AST levels (234–264 U/l), ALT levels (208–232 U/l), TGF- $\beta$  levels (1102–1265 pg/g liver tissue), hydroxyproline levels (633–719 nmol/g liver tissue), and liver fibrosis area (9.7–10.6 times for normal rat). These findings suggested that Sho-saiko-to extract is effective in the treatment of liver inflammation and fibrosis up to a certain degree of severity, but it produces no improvement in more severe cases.

**Key words** Sho-saiko-to; liver fibrosis; dimethylnitrosamine; retinoid; TGF- $\beta$ ; hydroxyproline

The mortality of patients with liver fibrosis is gradually increasing because liver fibrosis shows various pathologic conditions, which sometimes results in the development of liver carcinoma. In recent years, many studies have been performed to clarify the progression mechanism of liver fibrosis.

Sho-saiko-to extract has been used widely to treat chronic liver disease, including chronic hepatic inflammation and fibrosis in Japan. Sho-saiko-to is a Chinese herbal medicine prepared from seven herbs: Saiko (radix of *Bupleurum falcatum* L.), Hange (tuber of *Pinellia ternate* BREITENBACH), Wogon (radix of *Scutellaria baicalensis* GEORGI), Taiso (fructus of *Zizyphus vulgaris* LAMARCK var. *inermis* BUNGE), Ninjin (radix of *Panax ginseng* C. A. MEYER), Kanzo (radix of *Glycyrrhiza glabra* L. var. *glandulifera* REGEL et HERDER) and Shokyo (rhizoma of *Zingiber officinale* ROSCOE). Previously, we prepared model rats with liver fibrosis using bolus administration of DMN. Currently, hepatic fibrosis-inducing agents such as DMN are frequently used to experimentally induce liver fibrosis due to their convenience.<sup>1–23</sup> Then we reported that the liver fibrosis improving effect of Sho-saiko-to extract was due to the inhibition of activated myofibroblasts activation and enhancing liver cell regeneration.<sup>1–3</sup> However, the severity and state of liver inflammation and fibrosis vary among the respective animal models, which is problematic to clarify the relationship between liver conditions and drug effect. Therefore, we prepared model rats with various stages of liver injury by administering several dosages of DMN for different duration. Subsequently, Sho-saiko-to extract was administered to these rats, and its liver inflammation and fibrosis-improving effect was evaluated according to AST, ALT (serum biochemical indicators), TGF- $\beta$  (profibrogenic cytokine), liver retinoid levels (an index of fibroblast expression in the liver), levels of hydroxyproline (collagen biosynthesis indicator), and the liver fibrosis area levels (fibrosis indicator).

### MATERIALS AND METHODS

**Animals** Male Wistar rats, aged seven-weeks, 180–200 g, were purchased from NSC Japan (Shizuoka, Japan). Animals were acclimatized for seven days at 23±2 °C with free access of pellet food (CE-2, Clea, Osaka, Japan) and water. Healthy rats were then selected and seven animals assigned to each group.

**Materials** DMN, retinol palmitate and hydroxyproline were from Nacalai Tesque Inc. (Kyoto, Japan), pentobarbital was from Dai-Nihon Pharmaceuticals Inc. (Osaka, Japan). Sho-saiko-to extract was purchased from Tsumura drug manufactory (Tokyo, Japan). 4.5 g of dried Sho-saiko-to extract, which is prepared from boiled water extracts of seven herbs: 7.0 g of Saiko (radix of *Bupleurum falcatum* L.), 5.0 g of Hange (tuber of *Pinellia ternate* BREITENBACH), 3.0 g of Wogon (radix of *Scutellaria baicalensis* GEORGI), 3.0 g of Taiso (fructus of *Zizyphus vulgaris* LAMARCK var. *inermis* BUNGE), 3.0 g of Ninjin (radix of *Panax ginseng* C. A. MEYER), 2.0 g of Kanzo (radix of *Glycyrrhiza glabra* L. var. *glandulifera* REGEL et HERDER) and 1.0 g of Shokyo (rhizoma of *Zingiber officinale* ROSCOE). Sho-saiko-to powder was first dissolved in water and ethanol was gradually added to a final concentration of 80%. After centrifugation at 1400 g for 20 min, the resulting precipitate was discarded and supernatant was filtered through a 0.22- $\mu$ m filter and then evaporated to dryness on a rotary evaporator. The recovery of Sho-saiko-to extract after ethanol treatment was 54%. The dried extract was dissolved in water at a concentration of 50 mg/ml as a stock solution. The stock solution was then diluted in culture medium to the appropriate working solution.<sup>23</sup> For the presumed major active ingredients, the approximate concentrations of the components in 50 mg/ml of Sho-saiko-to extract were determined to be the following: glycyrrhizin, 500  $\mu$ g/ml (1.0%); baicalin, 1.75 mg/ml (3.5%); baicalein,

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150  $\mu\text{g/ml}$  (0.3%); each of saikosaponin-a, -c, -d, ginsenoside Rb1 and Rg1, 100  $\mu\text{g/ml}$  (0.2%); wogonin, 20  $\mu\text{g/ml}$  (0.04%); and Viscidulin III, less than 50  $\mu\text{g/ml}$  (<0.1%). All other chemicals were of reagent grade and used as received.

**Preparation of Liver Injury Model Rats** Liver injury model rats were produced by administration of DMN to the rats after acclimatization by the following methods. DMN was injected intraperitoneally. In the case of a single administration, the dose was 40 mg/kg. For daily administration (3 d), the dose was 10 or 15 mg/kg. For administration on every other day (3 times a week for 2 weeks), the dose was 10 or 15 mg/kg. As control, normal rats were examined.

**Administration of Sho-saiko-to Extract** Sho-saiko-to extract was administered in pellet food by adding the extract at concentration of 1.5% (5 times of clinical dose). After preparation of liver injury model rats by administration of DMN, they were allocated to either the Sho-saiko-to extract group or the ordinary food group. The Sho-saiko-to extract group was fed with 10 g per day of Sho-saiko-to-treated food (approximately 790 mg of Sho-saiko-to/kg) for 2 weeks from the 7th day after administration of DMN. The ordinary food group was fed similarly with ordinary food.

**Biochemical Examination** Blood samples were collected by sacrificing rats and serum levels of AST and ALT were measured using a Vision analyzer (Dainabot, Tokyo, Japan).

**Extraction and Measurement of TGF- $\beta$  in the Liver** Liver TGF- $\beta$  was extracted by the method described in the previous study,<sup>3)</sup> and measured using a multiplate spectrophotometer (Ultramark, Nihon Biorad, Japan) at a wavelength of 450 nm (reference, 570 nm).

**Measurement of the Hydroxyproline and Retinoid Lev-**

**els in the Liver** Liver retinoid levels were evaluated by measuring levels of retinol palmitate in the liver.

The hydroxyproline and retinoid levels in the liver were measured by the HPLC method as described in the previous studies.<sup>2,3)</sup>

**Histopathological Examination of the Liver** The area of liver fibrosis was determined according to the method of Yoshiji *et al.*<sup>24)</sup> Sections of the liver excised from the rats were produced and treated by Azan-mallory staining. Azan-mallory stained samples were processed by a computer using a Mac Scope, image analysis software (Mitani Shoji Inc. Japan), in 6 ocular fields (25 $\times$ magnification) per specimen of 6 rats.

**Statistical Analysis** Differences between groups were statistically examined by one-way analysis of variance. Where there was a significant difference, the means were compared by the Bonferroni method. Correlation coefficients were examined by the *t*-test.

## RESULTS

**Relationships between the Liver Inflammation or Fibrosis Parameters and DMN Administration Dose (Total Doses) in Liver Injury Rats** AST, ALT, liver TGF- $\beta$ , hydroxyproline levels and liver fibrosis area increased with increases in the total doses of DMN in a concentration dependent way. Liver retinoid levels decreased with increases in the total dose of DMN (Fig. 1). The body weight of the rats administered with DMN initially decreased approximately 5% on average due to loss of appetite. However, during the 2-weeks period of the experiment, there was no noticeable difference from control in the body weight of either the group

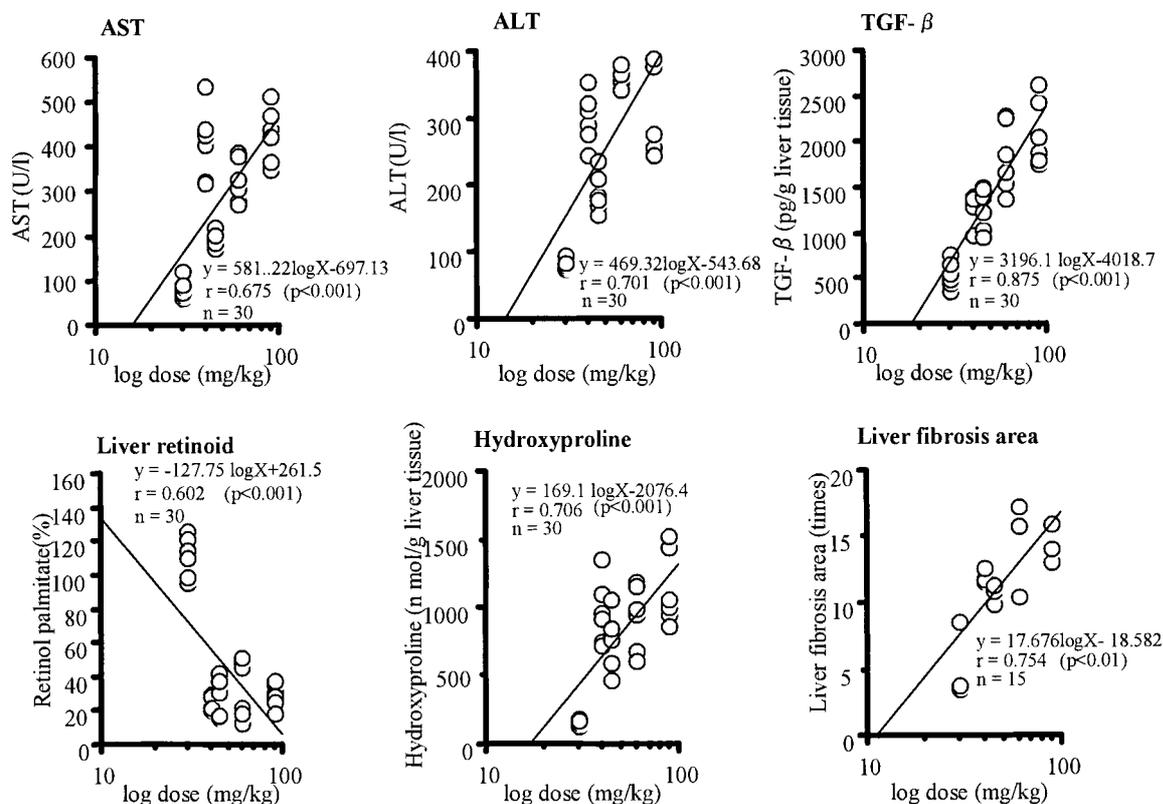


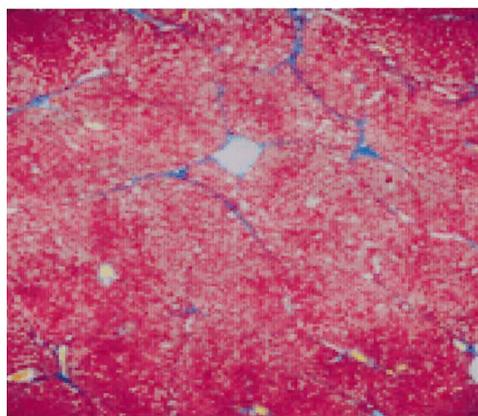
Fig. 1. Relationship between DMN Total Dose and Serum Biochemical Indices, Retinoid, TGF- $\beta$ , Hydroxyproline and Liver Fibrosis Area

Table 1. Effect of Administration of Sho-saiko-to Extract on Serum Biochemical Indicators in DMN Induced Liver-Injury Rats

Dosage	Dose of DMN (mg/kg)	Total dose of DMN (mg/kg)	AST (U/I)		ALT (U/I)	
			Ordinary food	Sho-saiko-to extract	Ordinary food	Sho-saiko-to extract
Control	0	0	98.0±35.0	111.0±41.0	31.0±5.0	37.0±8.0
1 time a day	40	40	408.0±78.0	203.0±46.0*	357.0±39.0	141.0±38.0*
1 time a day for 3 d	10	30	85.1±17.2	90.3±22.4	91.0±4.3	79.0±14.0
	15	45	216.9±25.1	164.6±15.7*	136.8±17.9	120.5±14.9
3 times a week	10	60	318.1±27.1	202.8±27.9*	379.3±20.3	205.1±18.1*
for 2 weeks	15	90	453.5±56.1	409.8±73.2	293.7±55.2	277.9±85.1

Each value is the mean±S.D. from six experiments. \* $p < 0.05$ , significantly different from the result of the Ordinary food group.

#### After administration of Ordinary food



#### After administration of Sho-saiko-to extract

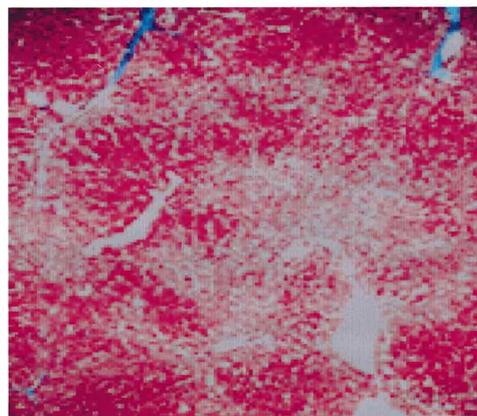


Fig. 2. The Influence of Administration of Sho-saiko-to Extract on the Azan-Mallory Stained Liver Tissue in DMN (40 mg/kg) Induced Liver-Injury Rats ( $\times 25$ )

fed with Sho-saiko-to extract or the ordinary food.

**Effect of Administration of Sho-saiko-to Extract on Serum Biochemical Indicators** When the rats were treated 40 mg/kg of DMN, 15 mg/kg of DMN daily for 3 d, or 10 mg/kg of DMN every other day three times a week for 2 weeks, AST levels in the Sho-saiko-to extract groups significantly decreased in a level approximately 49.8, 75.9, and 63.8% of those in the ordinary food groups. ALT levels in the Sho-saiko-to extract groups significantly decreased in a level approximately 39.5, 88.1, and 54.1% of those in the ordinary food groups. However, there were no significant differences in AST and ALT levels between the other Sho-saiko-to extract groups and the ordinary food groups (Table 1).

**The Influence of Administration of Sho-saiko-to Extract on the Liver Fibrosis Area** Figure 2 shows the Azan-Mallory stained liver tissue in rats. In rats treated with 40 mg/kg of DMN, the area of blue stained collagen fibers in the Sho-saiko-to extract groups was approximately 65% smaller than in the ordinary food groups.

**Effect of Administration of Sho-saiko-to Extract on TGF- $\beta$ , Liver Retinoid Levels, Hydroxyproline and Liver Fibrosis Area Levels in DMN Induced Liver-Injury Rats** When the rats were treated 40 mg/kg of DMN or 15 mg/kg of DMN daily for 3 d, TGF- $\beta$  levels in the Sho-saiko-to extract groups significantly decreased in a level approximately 66.5 and 80.7% of these in the ordinary food groups. However, there were no significant differences in TGF- $\beta$  levels be-

tween the Sho-saiko-to extract groups and the ordinary food groups when other regimens of DMN were used. When the rats were treated 40 mg/kg of DMN, retinoid levels in the Sho-saiko-to extract groups significantly increased to a level approximately 2.9 times higher than the ordinary food groups. However, there were no significant differences in retinoid levels between the Sho-saiko-to extract groups and the ordinary food groups when other regimens of DMN were used. When the rats were treated 40 mg/kg of DMN or 15 mg/kg of DMN daily for 3 d, hydroxyproline levels in the Sho-saiko-to extract groups significantly decreased in a level approximately 47.4 and 50.8% of the ordinary food groups. When other regimens of DMN were used, hydroxyproline levels in the Sho-saiko-to extract groups did not significantly differ from those in the ordinary food groups.

In the rats were treated 40 mg/kg of DMN or 15 mg/kg of DMN daily for 3 d, or 10 and 15 mg/kg of DMN every other day (three times a week) for 2 weeks, the liver fibrosis area in the Sho-saiko-to extract groups significantly decreased to a level approximately 64.7, 79.8, 65.8 and 62.9% of that in the ordinary food groups. However, there were no significant differences in the liver fibrosis area levels between the Sho-saiko-to extract groups and the ordinary food groups when other regimens of DMN were used (Table 2).

## DISCUSSION

Sho-saiko-to, one of the most widely used Chinese herbal

Table 2. Effect of Administration of Sho-saiko-to Extract on the TGF- $\beta$ , Retinol Palmitate, Hydroxyproline and Liver Fibrosis Area Levels in DMN Induced Liver-Injury Rats

Dosage	Dose of DMN (mg/kg)	Total dose of DMN (mg/kg)	TGF- $\beta$ (pg/g liver tissue)		Retinol palmitate (%)	
			Ordinary food	Sho-saiko-to extract	Ordinary food	Sho-saiko-to extract
Control	0	0	301.0 $\pm$ 131.2	336.7 $\pm$ 47.5	100	96.5 $\pm$ 4.3
1 time a day	40	40	1164.0 $\pm$ 207.0	774.0 $\pm$ 122.0*	21.3 $\pm$ 3.2	61.7 $\pm$ 10.4*
1 time a day for 3 d	10	30	373.6 $\pm$ 111.1	321.2 $\pm$ 128.5	105.0 $\pm$ 14.8	101.5 $\pm$ 9.7
	15	45	1106.2 $\pm$ 168.7	892.8 $\pm$ 130.1*	33.9 $\pm$ 13.4	41.4 $\pm$ 10.8
3 times a week	10	60	1875.8 $\pm$ 155.2	1615.5 $\pm$ 453.3	43.0 $\pm$ 18.0	39.9 $\pm$ 16.5
for 2 weeks	15	90	2006.2 $\pm$ 452.5	1992.8 $\pm$ 81.6	36.8 $\pm$ 9.0	28.2 $\pm$ 6.2

Dosage	Dose of DMN (mg/kg)	Total dose of DMN (mg/kg)	Hydroxyproline (nmol/g liver tissue)		Liver fibrosis area (times for normal rat)	
			Ordinary food	Sho-saiko-to extract	Ordinary food	Sho-saiko-to extract
Control	0	0	278.2 $\pm$ 11.0	304.6 $\pm$ 64.1	1	1
1 time a day	40	40	909.7 $\pm$ 204.5	431.1 $\pm$ 31.7*	11.6 $\pm$ 1.9	7.5 $\pm$ 1.1*
1 time a day for 3 d	10	30	175.8 $\pm$ 24.0	170.7 $\pm$ 22.5	5.9 $\pm$ 1.4	5.3 $\pm$ 2.8
	15	45	789.8 $\pm$ 252.1	401.2 $\pm$ 14.3*	10.4 $\pm$ 2.4	8.3 $\pm$ 1.2*
3 times a week	10	60	805.4 $\pm$ 154.6	757.8 $\pm$ 253.4	15.8 $\pm$ 2.0	10.4 $\pm$ 1.4*
for 2 weeks	15	90	994.3 $\pm$ 280.8	1064.1 $\pm$ 189.0	17.0 $\pm$ 2.6	10.7 $\pm$ 2.9*

Each value is the mean $\pm$ S.D. from six experiments. Liver retinoid concentration was expressed as a percentage of the liver retinoid level in normal rats measured shortly after the end of acclimatization, set as 100%. The times of the area of liver fibrosis of a normal rat, which was measured shortly after the end of acclimatization, was set as 1. \* $p$ <0.05, significantly different from the result of the Ordinary food group.

preparations, has long been used for the treatment of chronic liver diseases. We have already investigated its effect in retarding the process of liver fibrosis and accelerating liver regeneration, especially its effect on myofibroblasts that are thought to be deeply involved with liver fibrosis. As the results, we have found that Sho-saiko-to seems to be useful for repair of liver injury, improvement of fibrotic changes of the liver and promoting liver regeneration in liver-injured rats. This improvement seems to depend greatly on the suppression of activation of myofibroblasts in the liver, and the improvement of retinoid storage in the cells.<sup>1)</sup> To increase the liver retinoid level and decrease the hydroxyproline level or lower collagen production, in liver-injured rats.<sup>2)</sup> Moreover, Sho-saiko-to induces liver regeneration by increasing the production of HGF and suppressing the production of TGF- $\beta$  in the liver in liver-injured rats.<sup>3)</sup> In this time, we prepared model rats with various stages of liver injury by administering several dosages of DMN for different duration, to clarify the relationship between the stage of liver injury and effectiveness of Sho-saiko-to. We have already discussed the effects of various concentrations of extract (0.75, 1.5, 3.0%) on hepatopathy in rats in previous reports.<sup>1,3)</sup> According to the results of these previous experiments, we decided on the Sho-saiko-to extract concentration of 1.5%, which was administered in the present study. We have also carried out previous studies comparing the effects of Sho-saiko-to extract with glycyrrhizin, baicalin and baicalein, respectively. (Glycyrrhizin, baicalin and baicalein are the major active ingredients in Sho-saiko-to extract; especially glycyrrhizin is often administered clinically for hepatopathy.) The results of these studies showed the Sho-saiko-to extract is more effective than glycyrrhizin, baicalin and baicalein in the treatment of hepatopathy. In the DMN damage-induced rats, the liver inflammation and fibrosis parameters increased significantly and dose-dependently with the total dose of DMN compared to those in normal rats. DMN, which is a nitrosamine chemi-

cal used as an initiator of hepatopathy, is selectively and continuously accumulated in the liver. In the liver, drug metabolizing enzymes which activate the cytotoxicity of DMN and its metabolites are induced, resulting in hepatopathy.<sup>25,26)</sup> It was considered that the differences in the degree of liver fibrosis induced by DMN administration were resulted from different amounts of DMN and its metabolites in the liver corresponding to the amount of administrated DMN, causing different degrees of hepatopathy (AST and ALT levels). It was also considered that there was differences in the fiber expression process followed by hepatopathy, in which histological connection is repaired, (TGF- $\beta$  and retinoid levels), resulting in the differences in the final amount of collagen (hydroxyproline level and area of liver fibrosis). The amount of collagen was considered to increase with increases in the amount of administered DMN, *i.e.*, the cumulative amount of DMN, in a concentration-dependent way. Therefore, we suggested that the total dose of DMN was a useful index for predicting the severity of inflammation or fibrosis of the liver.

As for the amount of DMN administered, we also examined the effects of single administrations of 30 and 50 mg/kg (no data shown). As a result, the rats administered with a dose of 50 mg/kg all died within a week. In those administered with 30 mg/kg, AST and ALT levels were significantly higher than the control, but there were no significant differences shown by other indicators. We also examined the effects of Sho-saiko-to extract on rats whose DMN levels were over 60 mg/kg as a result of multiple administrations. In this case, we found no difference between Sho-saiko-to extract group and the ordinary food group. However, when a total dose of 40 or 45 mg/kg of DMN was used, Sho-saiko-to extract was found to improve DMN-induced liver fibrosis in rats. These findings suggested that Sho-saiko-to extract can improve DMN-induced liver fibrosis in rats when the severity of liver inflammation and fibrosis was limited within the following levels of the respective parameters: AST levels

(234—264 U/l), ALT levels (208—232 U/l), TGF- $\beta$  levels (1102—1265 pg/g liver tissue), hydroxyproline levels (633—719 nmol/g liver tissue), and the ratio of liver fibrosis area (9.7—10.6 times for normal rat). Moreover, Sho-saiko-to extract may not be useful for improving more advanced stage of liver inflammation and fibrosis such as cirrhosis. Therefore, it was considered that Sho-saiko-to extract should be administered to patients with mild inflammation and persistent fibrosis of the liver such as chronic hepatitis.

In the present study, we used liver retinoid levels as an index of the inhibition of myofibroblasts activation by Sho-saiko-to extract. Recently, it was reported that the activated myofibroblasts released retinoid outside the cells and produced various metabolites, thus accelerating the progression of liver fibrosis.<sup>27–29</sup> Therefore, the relationship between retinoid levels in the liver and the inhibition of myofibroblast activation by drugs should be evaluated by considering the influence of drug administration on retinoid metabolism.

In future, we will evaluate the liver-fibrosis improving effect of Sho-saiko-to extract in more detail in comparison with clinical application of Sho-saiko-to.

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