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XIAO-CHAI-HU TANG IN TREATING MODEL MICE WITH D-GALACTOSAMINE-INDUCED LIVER INJURY

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Abstract

This study explored the effects of a classical Chinese medicine formula- Xiao-Chai-Hu Tang(XCHT) on the model mice with D-galactosamine -induced liver injury. Sixty male imprinting control region (ICR) mice were used in the present study, and they were separated randomly into 6 groups: a normal control group (Group A, n=10), a model control (Group B, n=10), a positive control (Group C, n=10), a low dose of XCHT group (Group D, n=10), a medium dose of XCHT group (Group E, n=10), and a high dose of XCHT group (Group F, n=10). ELISA was used to detect the IL-6 and TNF-α levels in the serum. Real-time PCR was performed to assess the expression of FasmRNA, Fas-LmRNA, Bcl-2mRNA of the liver tissues. Western blotting was used to detect the Bax protein expression of the liver tissues. The serum IL-6 and TNF-α levels of Group B were significantly higher than the other groups ($P<0.05$). The expression of Fas mRNA, Fas-LmRNA, and Bax protein of the liver tissues of Group B were significantly higher than those of the other groups ($P<0.05$). The expression of Bcl-2 mRNA of the liver tissues of Group B was significantly lower than other groups ($P<0.05$). Both of XCHT and biphenyl dicarboxylate significantly decreased the serum IL-6 and TNF-α levels and FasmRNA, FasLmRNA, Bax protein expression and increased the Bcl-2 mRNA expression of the liver tissues of model mice ($P<0.05$). It may be through decreasing the serum IL-6 and TNF-α levels and FasmRNA, FasLmRNA, Bax protein expression and increasing the Bcl-2 mRNA expression of the liver tissues that XCHT significantly relieved the D-galactosamine -induced liver injury.

Key words: Chinese medicine formula, Xiao-Chai-Hu Tang(XCHT), Liver injury.

Introduction

Liver injury, is often caused by alcohol abuse, viral hepatitis, non-alcoholic steatohepatitis, autoimmunity and drug intoxication. In recent years, a number of drugs have been tested as anti-fibrogenic agents, but they generally do not suppress proliferation and collagen synthesis sufficiently (Kusunose et al, 2002). Therefore, it is necessary to find more

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potent agents for the treatment of liver injury and natural products derived from traditional Chinese herbal medicines may provide alternative treatment options for liver injury. An earlier study (Yamamoto et al, 1989) has however found that Xiao-Chai-Hu Tang (XCHT) extract is effective in the treatment of liver inflammation and fibrosis up to a certain degree of severity. In an earlier Chinese clinical study (Chen et al, 1990), 136 patients of HBsAg positive chronic hepatitis admitted from November 1986 to June 1988 were included, among them, 110 patients gave consent to have liver biopsy and out of the 110 liver biopsies 86 were confirmed histologically to be chronic active hepatitis. After screening the 86 patients twice with ELISA, 26 (30.23%) showed positive results twice. Though it was reported that the incidence of delta hepatitis infection is very low in the HBsAg carriers in Beijing area, the incidence of superinfection of delta hepatitis on chronic active hepatitis B seems to be considerably high as shown in this study. Histological examination revealed that in the liver of patients with superinfection of delta hepatitis on chronic active hepatitis (26 cases) there were more severe changes and more eosinophilic degeneration than in the liver of patients without superinfection (58 cases). The patients were allocated to 3 groups at random. Eleven cases of chronic active hepatitis, with superinfection were treated with Chinese traditional medicine XCHT, 5 cases with biphenyl dimethyl dicarboxylate and 10 cases with XCHT + biphenyl dimethyl dicarboxylate. It was noted that after 3 months of treatment, in the XCHT group, HBeAg became negative in 2/3, anti-HBe converted to positive in 2/8 and HBV-DNA converted to negative in 2/2. However, few researches have been conducted to detect the mRNA or protein changes of the relevant indicators. The present study was designed to explore the effects of the classic Chinese medicine formula-XCHT on the model mice with D-galactosamine -induced liver injury.

Material and methods

The Chinese medicine formula- XCHT was provided by the 1st Affiliated Hospital, Zhejiang University (Hangzhou, China). XCHT was made with 24g of *Bupleurum longiradiatum*, 9g of *Scutellaria pekinensis*, 9g of *Pinellia ternate*, 9g of *Zingiber officinale,* 9g of *Panax ginseng* and 6g of *Glycyrrhiza pallidiflora* and 6g of *Ziziphus jujube*. All the filtrates were combined, condensed and freeze dried. The extracted solution was stored at -20 ^{o}C. 60 male imprinting control region (ICR) mice (weighing 20 ±2 g) were provided by the Animal Laboratory Center, Zhejiang University (Hangzhou, China).The research was carried out according to the National Research Council's protocol for the care and use of laboratory animals. The mice were separated randomly into 6 groups: a normal control group (Group A, n=10), a model control (Group B, n=10), a positive control (Group C, n=10), a low dose of XCHT (Group D, n=10), a medium dose of XCHT (Group E, n=10), and a high dose of XCHT (Group F, n=10). Group A is normal mice with oral administration of saline. The rest 50 mice were randomly divided into five groups (Group B, C, D, E and F). Group B is model group with oral administration of saline. The mice in Group C were treated with oral administration of biphenyl dicarboxylate. Group D was treated with oral administration of 0.02g/kg of XCHT. Group E was treated with oral administration of 1g/kg of XCHT. Group F was treated with oral administration of 5g/kg of XCHT. These mice were orally administrated with medicine once per day for 14 days. Group B, C, D, E and F were induced into liver injury model with intraperitoneal injection of 400mg/kg of D-GalN 1 hr after the final oral administration of medicine. Biphenyl dicarboxylate purchased from Zhejiang Wang Bang Pharmaceutical Co.,

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Ltd. D-galactosamine (D-GalN, purchased from Ningbo Hi-Tech Biochemicals Co., Ltd.) was dissolved with saline and pH value adjusted to pH = 7. All the mice were sacrificed 24 hr after injection of D-GalN. The blood samples were taken from hepatic portal vein. The samples were centrifuged at 12,000 rpm for 10 min. The supernatant layer was taken for the ELISA assay of IL-6 and TNF-α. The liver specimens were frozen and stored at -80°C until the time of the assay. ELISA was used to detect the serum IL-6 and TNF-α levels. Real-time PCR was performed to assess the expression of FasmRNA, Fas-LmRNA, Bcl-2mRNA of the liver tissues. Western blotting was used to detect the Bax protein expression of the liver tissues. All the measurements were carried out in duplicate and were conducted according to the manufacturer's instruction.

Isolation of the total RNA was performed by using the RNAiso^{TM} Reagent (Haoji Bio-Tech, Hangzhou, China), according to the manufacturer's instructions. The purity and concentration of RNA were detected by NanoDrop®ND-100 Spectrophotometer (Thermo Fisher Scientific Inc, USA). Then cDNA was prepared from 500ng of total RNA by reverse transcription, using the PrimeScript^{TM} RT reagent Kit (Haoji Bio-Tech, Hangzhou, China). The cDNA samples were diluted in DNase- and RNase-free water at a proportion of 1:3 before further analysis. Quantitative real-time PCR was performed by using the iCycler iQ Real-Time Detection System (Bio-Rad). The Fas, Fas-L and Bcl-2 gene specific primers for human were provided by Sangon, Shanghai, China. Gene starting quantity was based on the cycle threshold (Ct) method. The control cDNA dilution series of known concentration were created for each gene to establish a standard curve, plotting the logarithm of the standard concentration against the Ct values. The samples were quantified from the measured Ct values by interpolation, using the regression equation. Each value was normalized to GAPDH, a housekeeping gene, to control for the amount of the input cDNA. The threshold cycle value for GAPDH mRNA was subtracted from that of the target gene, and the mRNA levels of the target gene were expressed as $2^{-\Delta Ct}$. The PCR Primers and conditions are shown as Table 1.

Table 1: Real-Time PCR Primers and Conditions

Gene	GenBank Accession	Primer Sequences	Size （bp）	Annealing (℃)
Mouse Fas	BC061160	5' CCAGACTTCTACTGCGATTCTCC 3' 5' CTGCAGTTTGTATTGCTGGTTGCT 3'	107	62
Mouse-FasL	BC052866	5' GTTCTGGTGGCTCTGGTTGGA 3' 5'GGTGTACTGGGGTTGGCTATTTG 3	143	63
Mouse-Bcl	NM_009741	5' GCTGGGATGCCTTTGTGGAACT 3' 5' CAGAGACAGCCAGGAGAAATCAAAC 3'	71	63
Mouse-18s	NR_003278	5' CGGACACGGACAGGATTGACA 3' 5' CCAGACAAATCGCTCCACCAACTA 3'	94	62

Bicinchoninic acid protein assay (Pierce, USA) was used to determine the protein concentration. Twenty μg of protein in loading buffer (final buffer composition: 50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS (w/v), 10% glycerol (v/v) and a trace amount of bromophenol blue) were incubated at 95°C for 5 min, cooled and then loaded per lane. Gel electrophoresis was performed on a Protean III mini-gel apparatus (Bio-Rad, Hercules, CA, USA) using 8% gel with 0.1% (w/v) SDS under a constant current of 35 mA and then transferred to nitrocellulose membranes for 1.5 hours. The membranes were blocked for 2 hrs at room temperature with 5% milk in Tris-Buffered Saline Tween. Membranes were incubated with primary antibody dilution (Bax antibody from Boster, 1:100; β-actin antibody from Santa Cruz, 1:2000) overnight at 4°C. After wash, the

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membranes were incubated with their corresponding secondary antibody at room temperature for 2 hrs. The proteins were detected with SuperSignal® West Dura Extended Duration Substrate and normalized against internal control β-actin.

The statistical analysis in the research was respectively conducted by three university-based postgraduates in our university. The data is expressed in mean±SD and tested by analysis of variance. For all hypothesis tests a 5% significance level ($p < 0.05$) and two-tailed tests were used. Ninety-five percent (95%) Mann-Whitney confidence intervals (CI) for the median difference between groups were determined.

Results and Discussion

Group serum comparison: IL-6 and TNF-α

As shown in Table 2, the serum IL-6 and TNF-α levels of Group B were significantly higher than all of the other groups ($P<0.05$). Both of XCHT and biphenyl dicarboxylate significantly decreased the serum IL-6 and TNF-α levels ($P<0.05$).

Table 2: Group serum comparison: IL-6 and TNF-α (U/L, Mean±SD)

Group	n	IL-6	TNF-α
A	10	11.04±2.54*	1.07±0.12*
B	10	58.45±2.40	72.10±4.77
C	10	48.93±2.43*	49.96±3.49*
D	10	50.10±1.99*	40.00±2.29**
E	10	39.61±2.35*	28.32±1.53*
F	10	37.66±2.36*	21.82±2.20*

Note: Compared with the model control group: *$P < 0.05$

Group mRNA comparison: Fas, Fas-L and Bcl-2

As shown in Figure 1, The expression of Fas mRNA and Fas-LmRNA of the liver tissues of Group B were significantly higher than all of the other groups ($P<0.05$), and the expression of Bcl-2 mRNA of the liver tissues of Group B was significantly lower than the other groups ($P<0.05$). Both of XCHT and biphenyl dicarboxylate significantly decreased the FasmRNA, FasLmRNA expression of the liver tissues of the model mice and increased the Bcl-2 mRNA expression of the model mice ($P<0.05$).

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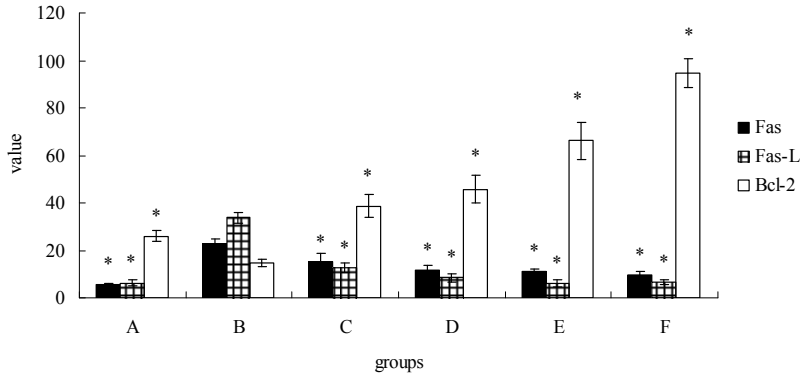

1 Group mRNA comparison: Fas, Fas-L and Bcl-2

Note: Compared with the model control group: *P < 0.05

Group Protein comparison: Bax

As shown in Figure 2, the Bax protein of the liver tissues of Group B was significantly higher than those of all the other groups (P<0.05). XCHT and biphenyl dicarboxylate both significantly decreased Bax protein expression of the liver tissues of the model mice(P<0.05).

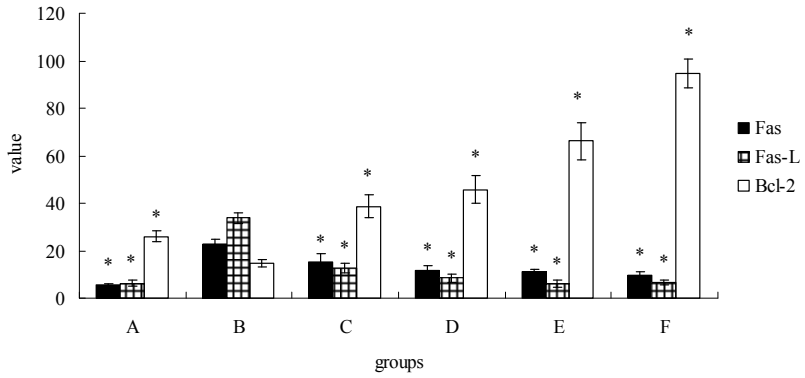

Figure 1: Group mRNA comparison: Fas, Fas-L and Bcl-2

Note: Compared with the model control group: *P < 0.05

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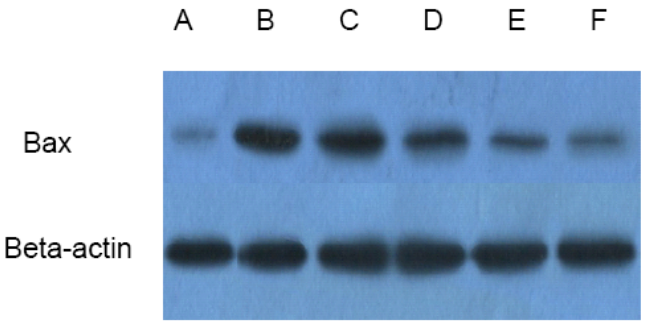

Figure 2: Group protein comparison: Bax

As early as 1989, a controlled prospective study (Chen et al, 1990) was conducted to evaluate the potential of XCHT for the prevention of hepatocellular carcinoma (HCC). Pairs of patients were matched for age, sex, presence of HBs antigen and the scores of the severity of liver dysfunction from 260 cirrhotic subjects. The researchers randomly assigned each patient to receive either a conventional medicine (control group), or 7.5 g/day of Syo-saiko-to (trial group). The patients were monitored during 34 months of treatment, and the incidence of HCC in the two groups were compared. Seventeen patients were found to have HCC in the control group, and nine were found to have HCC in the trial group. The incidence of HCC was significantly lower in the trial group. The results of this study suggested that XCHT may prevent or delay the emergence of latent HCC in patients with cirrhosis of the liver. XCHT is an important Chinese herbal prescription for curing many kinds of liver diseases (Chen et al, 2009). Other beneficial functionalities of XCHT reported in the recent literature include antioxidation, antimutagenesis, anticarcinogenesis, immunoregulation, and antiinflammation. A recent study (Nishimura et al, 2010) designed to investigate the effects of XCHT on the membrane permeability of tolbutamide in the intestinal tract found that it might facilitate the energy-dependent transport of tolbutamide across the rat jejunum in-situ and Caco-2 cell monolayers.

Administration of XCHT was associated with preventive and beneficial effects against various types of drug- or chemical-induced liver damage. XCHT has been found effective in treating liver inflammation and fibrosis (Kusunose et al, 2002). The enhanced activity of natural killer (NK) cells in the liver was found to be involved in the process. (Kaneko et al, 1994). Another proposed mechanism is a dose-dependent increase in the production of granulocyte colony-stimulating factor (G-CSF) on peripheral blood mononuclear cells (Yamashiki et al, 1992). The study demonstrated that the classic Chinese medicine formula--XCHT showed promise in relieving liver injury and merits further study. To conclude, it may be through decreasing the serum IL-6 and TNF-α levels and FasmRNA, FasLmRNA, Bax protein expression and increasing the Bcl-2 mRNA expression of the liver tissues that XCHT significantly relieved the D-galactosamine -induced liver injury. Further research with larger samples needs to be conducted on the pathway related mechanism involved.

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References

1. Chen, N.L., Gu, F.and Jia, K.M.Chronic active hepatitis with superinfection of delta virus and hepatitis B virus: treatment with Chinese traditional medicine. Zhonghua Nei Ke Za Zhi. 1990;29(3):144-146, 189.
2. Chen, F.P., Chen, F.J., Jong, M.S., Tsai, H.L., Wang, J.R. and Hwang, S.J., (2009). Modern use of Chinese herbal formulae from Shang-Han Lun. Chin Med J (Engl). **122(16)**:1889-1894.
3. Kaneko, M., Kawakita, T., Tauchi, Y., Saito, Y., Suzuki, A. and Nomoto, K., (1994). Augmentation of NK activity after oral administration of a traditional Chinese medicine, xiao-chai-hu-tang (shosaiko-to). Immunopharmacol and Immunotoxicol. **16(1)**:41-53.
4. Kusunose, M., Qiu, B., Cui, T., Hamada, A., Yoshioka, S., Ono, M., Miyamura, M., Kyotani, S. and Nishioka, Y., (2002). Effect of Sho-saiko-to extract on hepatic inflammation and fibrosis in dimethylnitrosamine induced liver injury rats. Biol Pharm Bull. **25(11)**:1417-1421.
5. Nishimura, N., Uemura, T., Iwamoto, K. and Naora, K., (2010). Change in tolbutamide permeability in rat jejunum and Caco-2 cells by Sho-saiko-to (Xiao Chai Hu Tang), a Chinese traditional medicine. J Pharm Pharmacol. **62(5)**:651-657.
6. Yamamoto, S., Oka, H., Kanno, T., Mizoguchi, Y. and Kobayashi, K., (1989). Controlled prospective trial to evaluate Syosakiko-to in preventing hepatocellular carcinoma in patients with cirrhosis of the liver. Gan To Kagaku Ryoho. **16(4 Pt 2-2)**:1519-1524.
7. Yamashiki, M., Asakawa, M., Kayaba, Y., Kosaka, Y. and Nishimura, A., (1992). Herbal medicine “sho-saiko-to” induces in vitro granulocyte colony-stimulating factor production on peripheral blood mononuclear cells. J Clin Lab Immunol. **37(2)**:83-90.