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RAPID COMMUNICATION

Antiviral effect of Chinese medicine jiaweisinisan in hepatitis B virus transgenic mice

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Abstract

AIM: To study the antiviral effect of Chinese medicine jiaweisinisan (JWSNS) on hepatitis B virus (HBV) infection in transgenic mice (TGM).

METHODS: Twenty two 6-8 wk old HBV TGM in the third generation were divided into TGM control group and TGM treated group randomly. The normal control group included ten normal BC 57L/6 mice at the same age. The mice in treated group were administrated with JWSNS at the concentration of 4 g/mL and the dosage of 50 g/kg per d for 30 d, while the mice in TGM control group and normal control group were administrated with normal saline at the same dosage and the same time. Polymerase chain reaction (PCR) was used to assess the contents of HBV DNA in serum of HBV TGM before and after treatments, whereas blot hybridization was utilized to measure the contents of HBV DNA in the liver of both HBV TGM and normal BC 57L/6 mice.

RESULTS: The levels of serum HBV DNA in TGM treated group were remarkably decreased after the treatment of JWSNS (7.662 ± 0.78 *vs* 5.22 ± 3.14, $P < 0.05$), while there was no obvious change after administration of normal saline in TGM control group (7.125 ± 4.26 *vs* 8.932 ± 5.12, $P > 0.05$). The OD values of HBV DNA in the livers of the mice in TGM treated group were significantly lower than those of TGM control group (0.274 ± 0.096 *vs* 0.432 ± 0.119, $P < 0.01$).

CONCLUSION: JWSNS exerts suppressive effects on HBV DNA in the serum and liver of TGM.

Key words: Traditional Chinese herbs; Hepatitis B virus; Transgenic mice; PCR; Blot hybridization; Chronic hepatitis B

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INTRODUCTION

In this *in vivo* study, HBV transgenic mice (HBV TGM) models were established to detect the antiviral effects of traditional Chinese medicine, jiaweisinisan (JWSNS) on HBV TGM, so to further confirm the inhibitory effects of this traditional Chinese herb on HBV infection.

MATERIALS AND METHODS

Experimental animals

Normal C57BL/6 mice and the HBV transgenic mice, living in the same cote, were provided by Department of Transgenic Engineering in Hepatopathy Research Center of Guangzhou Military Hospital. All the non-transgenic mice were under close surveillance to ensure the HBV DNA in serum and tissue to be negative.

Traditional Chinese herbs

JWSNS, including buplerum chinense DC, flea body, prunus persica (L.) batsch, of 10 grams each, and radix paeoniae alba, fructus aurantii immaturus, dipsacus asper wall, rhizoma dryopteris crassirhizomae, eupatorium adenophorum sprengel, of 12 grams each, as well as 5 grams of glycyrrhizaglabral, and 30 grams of loranthus parasiticus, was prepared according to the traditional procedures. Five compounds of drugs were mixed together, 141 grams per compound, and dissolved into the water to distil twice, yielding 1500 mL distillation solution, followed by inspissation of the distillation to 180 mL. The final concentration was 4 g/mL, stored in refrigerator for use.

PCR primer and reagent

PCR primers and relevant reagents were provided by

Shanghai Bioengineering Research Center of Chinese Academy of Sciences. The sequence of PCR primer one is 5'-TGGCACTAGTAAACTGAGCC-3' and that of PCR primer two is 5'-ACATCAGGATTCCTAGGACC-3'. Other reagents such as $MgCl_2$, dNTP, buffer, Tag enzyme, and paraffin oil were purchased from Promega Company (Madison, USA). Quantitative diagnostic kit (batch number 1000-902-1) for HBV DNA was provided by Biotromcs Technological Company (San Francisco, USA).

DNA extraction kit

DNA extraction kit was obtained from Maikang Biotechnological Company of Zhongshan Medical University.

Recombinant plasmid PBR322-2.0 HBV rapid extraction reagents

The reagents included host strain, antibiotics, peptone, yeast extract, gelose, buffer Ⅰ (50 mmol/L glucose, 25 mmol/L Tris HCl, 10 mmol/L EDTA), buffer Ⅱ (0.2 mol/L NaOH, 1% SDS), and buffer Ⅲ (5 mol/L potassium acetate 60 mL, iced acetic acid 11.5 mL, water 28.5 mL).

α-^{32}P-DNA probe labeled reagents

It included NEN kit (Promega Co., Madison, USA), Sephaclex G-50 columniation, purified recombinant plasmid PBR322-2.0 HBV (100 ng/μL), 0.5 mol/L EDTA.

Blot hybridization reagents

It included nitrate fibrous membrane (aperture 0.45 μm, Amersham Co., Buckinghamshire, UK), Formamide 20×SSC buffer, 37% formaldehyde, dyestuff (25% Bromophenolblue dissolved into Ficoll), TE buffer.

Apparatus

Gene amp PCR system (Techne, England), AG-9600 equipment for analytical fluorescence microscope (USA), low-temperature freeze centrifuge (Biofuge 22R, Germany), water bath, pH meter, magnetic shaker, vacuum pump, refrigerator, balance (China), constant temperature rocker, constant temperature incubator, superclean working table (China), ZXZ-4 gyral vacuum pump (Zhejiang Linhai Vacuum Apparatus Factory), 721 spectrophotometer (The Third Spectrophotometry Factory, Shanghai) were used.

Establishment of HBV TGM model

P2.0 HBV plasmid passed through a series of *Eco*RI/*Sal*I restriction enzyme digestion, and electrophoresis, followed by the collection of 7.0 kb DNA fraction, which contained two end-to-end 3.2 kb HBV entire genes, and 0.6 kb PBR322 DNA vector. After electrophoresis quantitative analysis, the collected DNA fractions were dissolved in TE buffer under bioclean condition to ensure the concentration to be 1 mg/L, then respectively packed and stored at -20 ℃ to wait for micro- injection. C57BL/6 mice injected entire genome plasmid which contained HBV were identified to be G0 generation. Positively integrating male mice of G0 generation were selected to hybridise with infraspecific normal female mice, producing G1 generation. In similar manner, positively integrating male mice of G1 generation were selected to copulate with infraspecific normal female mice, producing G2 generation. Mice of G3 generation were produced in the same way[1].

Selecting procedures of HBV TGM

A total of one hundred and fifteen 6-8 wk old mice of G3 generation, weighing 20 g, were provided by Department of Transgenic Engineering in Guangzhou Military Hospital. HBV DNA detection kit was purchased from Baosheng Bioengineering Company (Dalian). Tissues were firstly detected to select HBV DNA positive mice, followed by serum detections. Twenty-two serum HBV DNA positive mice were eventually selected from 115 mice of G3 generation to be labeled as HBV DNA transgenic mice.

Grouping

Twenty-two HBV TGM, whose serum HBV DNA was positive, and 10 normal C57BL/6 mice were grouped into TGM treated group, 12 TGM mice; TGM control group, 10 TGM mice; normal control group, 10 normal C57BL/6 mice; and then registered. Additional six 6–8 wk old C57BL/6 mice, weighing (20 ± 2) g, whatever male or female, were also prepared. All the mice were fed in Experimental Animal Center of Guangzhou Military Hospital. Auto-supply of water and standard food were offered as well as air condition to maintain the constant temperature during the whole experimental procedures.

Administration

Mice in TGM treated group were administrated with JWSNS at 50 g/kg per d, in 0.3-0.4 mL JWSNS solution (about 4 g/mL, ig) once a day for four weeks, while the mice in the other two control groups were administrated with the same dosage of normal saline at the same time.

Sample collection

Two hours after the last administration, eyeballs were extirpated to collect peripheral blood before the execution of the mice followed and sera were then separated for detection of HBV markers. Promptly, livers were removed and broken, frozen by liquid nitrogen and stored at -70 ℃ for detection.

Detection method

PCR quantitative analysis[2, 3] was used to detect the serum contents of HBV DNA of TGM at the moment when the total contents had reached up to 1.0×10^5 kb/mL, while blot hybridization[4, 5] was utilized to analyze the contents of HBV DNA in liver of mice. The extraction of DNA in liver tissue was processed according to the instruction provided by the manufacturer of the kit. The rapid extraction of recombinant plasmid PBR322-2.0 HBV was performed by alkali fission method[6]. α-^{32}P-DNA probe was labeled according to instructions of Promega Co. reagent kit. Blot hybridization was performed as follows: 40 μL α-^{32}P-DNA probe labeled solutions was used

Table 1 Changes of serum levels of HBV DNA in HBV TGM before and after treatment of JWSNS (mean±SD)

Group		*n*	Serum HBV DNA	
			Case transformed from DNA positive to negative	Content of HBV[1]
TGM controlled group	Prior to administration of normal saline	10	0	7.125±4.26
	After administration of normal saline	10	0	8.932±5.12
TGM treated group	Prior to JWSNS treatment	10	0	7.662±0.78
	After JWSNS treatment	10	3	5.122±3.14

[1]Analyzed by *t*-test. TGM treated group, [1]$P<0.05$ *vs* TGM controlled group, [1]$P>0.05$.

Table 2 Effect of JWSNS on levels of HBV DNA in the liver of HBVTGM (mean±SD)

Group	*n*	HBV DNA blot hybridization
Normal control group	9	0
TGM control group	9	0.432±0.119
TGM treated group	9	0.274±0.096[b]

[b]$P<0.01$ *vs* TGM control group.

to dot on the membrane, which was then dipped in the metamorphic solution, and baked in the oven at 80 ℃ for two hours. After pre-interaction, membrane washing, slice nipping, the positive degree was justified according to the OD value of each blot.

Statistical analysis

Statistical data was analyzed by SPSS software. The comparability prior to and after disposal of the same sample was verified by paired-samples *t*-test, whereas the difference of the mean value among various groups was analyzed by χ^2 test. *P* less than 0.05 was taken as significant.

RESULTS

Effects of JWSNS on serum contents of HBV DNA in HBVTGM

The levels of serum HBV DNA in TGM treated group displayed considerable distinction before and after treatment of JWSNS ($P<0.05$), compared with those in TGM controlled group in which no significant difference was shown before and after administration with normal saline ($P>0.05$) (Table 1).

Effect of JWSNS on levels of HBV DNA in the liver of HBV TGM

HBV DNA blot hybridization was positive in HBV TGM both before and after treatment. The OD value showed extremely significant difference between TGM controlled group and TGM treated group ($P<0.01$), whereas blot hybridization in normal control group appeared to be negative (Table 2).

DISCUSSION

Value of HBV TGM model in the study of inhibitory effect of the traditional Chinese medicine on HBV

The host infected by HBV showed violent phyletic and tissue specificity, resulting in the remarkable restriction in the establishment of the animal model infected by HBV and anti-HBV study. In the past, data related to anti-HBV entirely came from HBV-infected patients, orangutan or cell *in vitro*. In addition, significant distinction existed between experimental data of other hepatophilic DNA virus such as duck or groundhog HBV and those of human HBV[7,8].

In 1980, microinjection HBVTGM model was successfully established which obviously overcame the limitation described above. Chissari [9] established TGM model and claimed that HBV DNA and HBsAg granules were detected in TGM blood and congregated in a fraction of hepatocyte, inducing tumefaction and dysfunction of the endoplasm as well as the ground glass hepatocytes, besides enhancing the sensitivity of hepatocytes to the lipopolysaccharide and IFN-γ, resulting in the damnification, necrosis and regeneration of the hepatocyte, or even the occurrence of hepatocarcinoma. Thus, HBVTGM model was, to a great extent, similar to the immune interaction between virus and host during natural infection of HBV[10].

HBVTGM is viewed as an immune tolerance condition. Despite limited damnification of hepatocytes in some mice, inflammation was not obvious, which is consistent with the pathologic change in human beings infected with HBV. The objective of our study emphasized on suppressive effect of JWSNS on HBV including the change of HBV contents in blood and tissue rather than the inflammatory level in liver. Thus, it is appropriate that HBVTGM served as the anti-HBV model.

Anti-HBV effect of traditional Chinese medicine and significance of HBV DNA detection

The symptom in different stage of HBV persistent infection varies from chronic asymptomatic HBV carrier and chronic hepatitis to hepatocirrhosis and hepatocarcinoma[11,12]. According to modern medicine chronic asymptomatic HBV carrier is viewed as the earlier stage of chronic persistent infection of HBV, in which the immune system is inhibited, leading to the inefficacy of interferon and lamivudine, the traditional antivirus drugs. Thus it is a widespread viewpoint that there is no effective treatment for earlier stage of HBV infection. However, it does not mean that there is no need for treatment[13,14]. Of note, at this stage, viruses copy themselves constantly, accompanied with the obvious viraemia, and approximately

normal liver function. However, as the copying process continues, a series of immune reaction of the host would be triggered to damage the liver and other organs. This stage may be a chance for traditional Chinese medicine to act as antivirus agent[15]. Pioneering clinical experiences indicated that traditional Chinese medicine such as phyllanthus urinaria L., matrine could endow the host in immune tolerance condition advantage in antivirus effect and protective effect on liver, and the detection of HBV DNA contents could help evaluate the antivirus effect of traditional Chinese medicine.

Theoretic basis for treatment of HBV infection by JWSNS

The treatment regimen of chronic hepatitis B by JWSNS is rooted in pathologic hypothesis of traditional Chinese medicine proposed by Bao Yi Liu, a famous doctor in ancient China who stated that whenever the *nephric qi* is inadequate, the protective function of human body against disease would decline, whereas *sick qi* would take the chance to invade the human body, weakening the *hepatic qi*, inducing the damp and the heat, which then attack the spleen and stomach, leading to the disfunction of both liver and spleen. This main pathologic process persists during the whole course of disease. According to this pathologic hypothesis, nothing but nourishing *nephric qi* is the key step to cure chronic hepatitis B. JWSNS, a famous compound, is used to enrich *nephric qi,* thus to reinforce the protective effect of the human body, and to overcome the state of immune tolerance.

Inhibitory effect of JWSNS on HBV of HBVTGM

In this study, HBVTGM model was used to observe the change of HBV DNA content both in serum and in hepatic tissue before and after the JWSNS treatment. The contents of HBV DNA in liver reflect the contents of HBV in hepatocyte. HBV, a hepatophilic virus, invades into the hepatocyte, in which they copy themselves, and then migrate into the peripheral circulation, inducing the diffuse chronic infection of HBV. The contents of HBV DNA reflect the level of virus copy. In the study, hepatocellular DNAs were extracted, and with the probe of P^{32} labeled plasmid P2.0 HBV, blot hybridization proceeded in nitrate fibrous membrane. The results of blot hybridization showed that no blot could be seen in normal control group, in striking contrast with the obvious blot appearing in both TGM control group and TGM treated group. The measurement of OD value of the blot demonstrated that the contents of HBV DNA in hepatocyte dramatically decreased four weeks after JWSNS treatment, compared with HBVTGM control group ($P < 0.01$).

PCR, a comparatively sensitive method was utilized in detecting the change of serum HBV DNA before and after JWSNS treatment in treated group or before and after administration of normal saline in control group. Four weeks later, no significant change could be seen in TGM control group administrated with normal saline, whereas HBV DNA of three mice was converted from positive to negative after treatment with JWSNS, and the serum contents of HBV DNA showed significant decline before and after treatment ($P < 0.05$).

The detection of HBV DNA contents in both liver tissue and serum shows that JWSNS could, to certain extent, inhibit HBV DNA, which provides the experimental proof for treatment of chronic hepatitis B with JWSNS.

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