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Cimicifuga foetida L. plus adefovir effectively inhibits the replication of hepatitis B virus in patients with chronic hepatitis B

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Abstract. The aim of the present study was to assess the anti-hepatitis B virus (HBV) effect of *Cimicifuga foetida* L. (*C. foetida*) in the patients with chronic hepatitis B (CHB). A total of 60 randomly selected patients with CHB were recruited and divided into groups I and II. The patients in group I received a monotherapy of adefovir (ADV), and the patients in group II received a combination therapy of ADV and *C. foetida* for >48 weeks. Intrahepatic (IH) HBV covalently closed circular DNA (cccDNA), serum HBV DNA, hepatitis B surface antigen (HBsAg), alanine aminotransferase levels and serum interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) levels were quantified during the test. Following the treatment, a significant reduction of the median IH cccDNA level was identified in group II (P=0.017), but not in group I (P=0.05, and P=0.01 between the 2 groups), and a significant reduction of $\log_{10}$ HBsAg was identified in groups I (P=0.012) and II (P<0.0001, and P=0.20 between the 2 groups). A significant increase of the median serum IFN-γ level was found in group II (P=0.0005), but not in group I (P=0.06, and P=0.004 between the 2 groups), and a significant reduction of the median TGF-β level was identified in groups I (P<0.0001) and II (P<0.0001, and P=0.002 between the 2 groups). A total of 24 patients in group I, and 27 patients in group II achieved a sustained virological response (P=0.0386), and 20 patients in group I and 24 in group II achieved hepatitis B e antigen seroclearance (P=0.0442). In conclusion, *C. foetida* can effectively inhibit HBV transcription and replication in the patients by stimulating the release of the inflammatory cytokines, such as IFN-γ.

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Introduction

Hepatitis B virus (HBV) infection remains one of the most serious global health problems. Conservative estimates of the global prevalence of HBV infection approximate that the number of affected individuals is >2 billion worldwide, among which the infection may develop into chronic hepatitis B (CHB) in ~240 million people. As a non-cytopathic virus, HBV infection may induce the host immune responses, producing substantial liver damage and resulting in chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). Covalently closed circular DNA (cccDNA), generated from the partially double-stranded genomic DNA (relaxed circular DNA) in the nucleus of infected hepatocytes, represents the transcriptional template for HBV RNA production and has a significant role in the persistence of HBV infection, the infection of liver transplant cases (2) and the pathogenesis of HCC (3). To date, the treatment with nucleoside analogues, such as lamivudine, adefovir (ADV) and entecavir, either as a monotherapy or with interferon-α (INF-α), has been the major intervention capable of eradicating HBV from infected cells (4). However, long-term nucleoside analogue monotherapy may not eradicate cccDNA in the nucleus directly and completely, and may result in HBV infection relapse due to the continuous emergence of drug-resistant mutation. The efficacy of INF-α is limited to a small percentage of highly selected patients and is often associated with adverse effects, such as flu-like symptoms, fatigue, leucopenia, depression, anorexia and hair loss (5).

In China, numerous herbs or their derivatives have also been widely used in the treatment of HBV infection. *Cimicifuga foetida* L. (*C. foetida*), which mainly consists of *C. foetida*, Kudzuvine root, Chinese herbaceous peony and liquorice, has been used as a medical plant for anti-pyretic and detoxificative purposes in ancient China for thousands of years, and has been proved to have anti-bacterial, anti-inflammatory, anti-pyretic and anticancer activities (6). *C. foetida* has also shown a promising anti-HBV effect in CHB patients, and has been approved for the treatment of HBV infection by the State Food and Drug Administration of China (7). Thus far, however, the anti-HBV effect of *C. foetida* has not yet been characterized *in vivo* and *in vitro*. In the present study, the inhibition of HBV replication, particularly at the cccDNA level during the combination therapy of ADV and *C. foetida*, was compared to the monotherapy of

ADV, and demonstrated that *C. foetida* may stimulate the release of the inflammatory cytokines, such as IFN-γ, and possibly inhibit the function of the immunosuppressive cytokines in the therapy, such as transforming growth factor-β (TGF-β).

Patients and methods

Patients and samples. A total of 60 randomly selected patients with treatment-naive, new diagnosed CHB, from the Department of Infectious Diseases, Renmin Hospital, Hubei University of Medicine (Shiyan, China), were recruited and divided into 2 groups: Group I comprised of 30 patients and group II comprised of 30 patients, respectively. CHB was documented by the presence of HBV DNA in the serum for >6 months and a serum alanine aminotransferase (ALT) level greater than twice the normal range (4). Patients who were coinfected with hepatitis D, hepatitis C or human immunodeficiency virus, or those with Wilson's disease or primary biliary cirrhosis were excluded from the study. While the patients in group I received a monotherapy of ADV (10 or 30 mg daily) for >48 weeks, the patients in group II received a combination therapy of ADV and *C. foetida*. Briefly, 10 g of *C. foetida*, 10 g of Chinese herbaceous peony and 10 g of liquorice, and 15 g of Kudzuvine root were shade-dried and decocted together for 1 h with 1 liter of boiling reverse-osmotic water twice. The decoctions were mixed, filtered, concentrated and lyophilized, and the dose of which (100 ml) was taken three times a day (8). Each patient signed an informed consent document approved by the Ethics Committee of Shiyan People's Hospital (Shiyan, China). Liver biopsy specimens were collected from each patient at baseline and week 48, were frozen in liquid nitrogen and were stored at -70˚C until experimental analysis. Serum samples were collected simultaneously and stored at -70˚C until used for the measurement of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and ALT levels.

Intrahepatic (IH) HBV cccDNA quantification. DNA was extracted from biopsy specimens using the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany). Determination of IH HBV cccDNA levels, prior and subsequent to the treatment, was carried out by quantitative polymerase chain reaction, as described previously with a slight modification (9). Variation in the amounts of liver tissue was normalized by quantifying β-globin in each sample with the Roche DNA control kit to allow standardization of the extracted DNA and expression of HBV cccDNA as copies per cell (copies/cell).

Assays for serum HBV DNA, HBsAg, HBeAg and ALT. DNA was extracted from 200 μl serum using the QIAamp® DNA Blood Mini kit (Qiagen). Serum HBV DNA levels were measured using the Cobas® TaqMan® test as described previously (Roche Diagnostics, Indianapolis, IN, USA) (10). Serum HBsAg and HBeAg levels were quantified through enzyme immunoassays (ARCHITECT platform; Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's protocol. The lower limits of the detection were 15 IU/ml for HBV DNA, 0.05 IU/ml for HBsAg and 1 IU/ml for HBV DNA, respectively. During the study, the serum HBV DNA and HBsAg levels were detected every 3 weeks. Serum ALT was measured prior and subsequent to the treatment using the ALAT (GPT) FS kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) according to the manufacturer's protocol.

Table I. Baseline patient characteristics.

Characteristics	Group I (n=30)	Group II (n=30)	P-value
Age, years	43 (41-56)	45 (35-55)	0.2
Male	24	25	1.0
Serum ALT, IU/l	220 (120-390)	225 (118-450)	0.6
Serum HBV DNA, log copies/ml	8.2 (6.3-8.9)	6.8 (5.8-8.1)	0.1

Data presented as mean (range) or n (%). ALT, alanine aminotransferase; HBV, hepatitis B virus.

Serum IFN-γ and TGF-β quantification. Serum IFN-γ and TGF-β levels were quantified using ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) prior and subsequent to the treatment. The A450 nm was determined with the ELISA reader (Multiskan EX; Thermo Labsystems, Helsinki, Finland).

Statistical analysis. Continuous variables from the treatment groups are expressed as the mean (range) and were analyzed using a non-paired Student's t-test using statistical software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). HBV DNA (IU/ml) and HBsAg (IU/ml) were logarithmically transformed for analysis. The Kaplan-Meier method (using a log-rank test) was applied for the cumulative rates of sustained virological response (SVR) (11) and HBsAg seroclearance. Differences were two-sided and P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics of patients. The baseline characteristics of the patients prior to treatment are shown in Table I. All the patients were positive for HBsAg and HBeAg ≥6 months, with a serum ALT level greater than twice the normal range.

IH HBV cccDNA, serum HBsAg, IFN-γ and TGF-β quantification. The lower limits of detection for IH cccDNA were 2.4×10^{-4} copies/cell. IH cccDNA levels were detectable in all patients prior to the treatment, and in 27 patients in group I and 25 in group II following the treatment. The median IH cccDNA level was 3.82 copies/cell in group I and 3.75 copies/cell in group II, respectively, prior to the treatment (P=0.68). Following the treatment, a significant reduction of the median IH cccDNA level was found in group II (0.15 copies/cell, P=0.017) but not in group I (0.76 copies/cell, P=0.05), which was significantly lower in group II (P=0.01). Prior to the treatment, $\log_{10}$ HBsAg was 3.52 in group I and 3.71 in group II, respectively (P=0.26). Following the treatment, 2 patients in group I, and 4 patients in group II had achieved HBsAg seroclearance, and a significant reduction of $\log_{10}$ HBsAg could be found both in group I (2.78, P=0.012) and in group II (2.27, P<0.0001), and there was no significant difference identified between the 2 groups (P=0.20). Prior to the treatment, the

Table II. Virological and serological responses following 48 weeks of anti-viral therapy.

Assays	Group I	Group II	P-value
Mean IH cccDNA, copies/cell (range)			
Prior to treatment	3.82 (0.035 to 42.2)	3.75 (0.049 to 37.6)	0.682
Subsequent to treatment	0.76 (neg to 4.42)	0.15 (neg to 2.08)	0.013
P-value	0.052	0.017	
Mean serum IFN-γ, pg/ml (range)			
Prior to treatment	14.8 (8.6 to 24.4)	15.3 (9.2 to 26.4)	0.291
Subsequent to treatment	17.1 (9.1 to 27.7)	20.9 (9.3 to 36.9)	0.004
P-value	0.063	0.0005	
Mean serum TGF-β, pg/ml (range)			
Prior to treatment	672.9 (495.5 to 831.8)	630.5 (428.7 to 921.9)	0.151
Subsequent to treatment	338.9 (253.4 to 450.1)	286.0 (135.9 to 519.7)	0.002
P-value	<0.0001	<0.0001	
Mean serum HBsAg, log IU/ml (range)			
Prior to treatment	3.52 (2.14 to 4.90)	3.71 (2.27 to 4.69)	0.263
Subsequent to treatment	2.78 (-2.04 to 3.48)	2.27 (-2.02 to 3.41)	0.201
P-value	0.012	<0.0001	

IH, intrahepatic; cccDNA, covalently closed circular DNA; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; HBsAg, hepatitis B surface antigen.

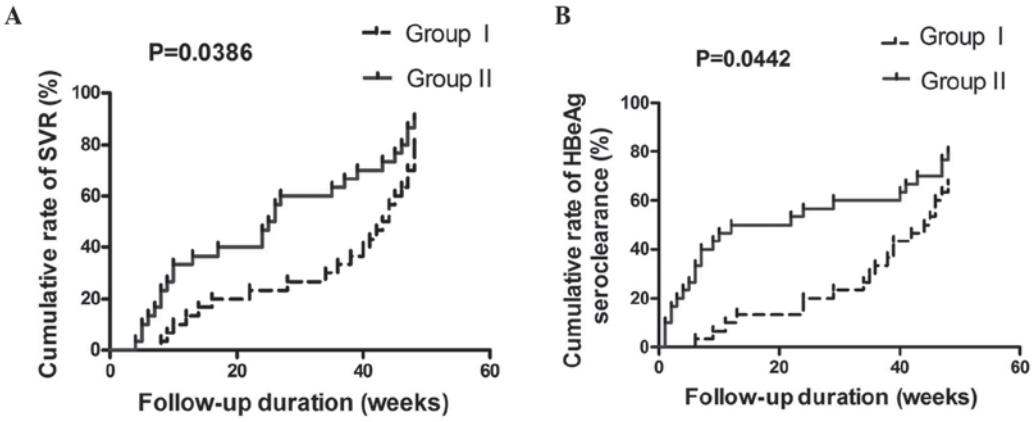

Figure 1. Differences of the cumulative rate analysis between groups I and II. Cumulative rate analysis (A) of sustained virological response (SVR) and (B) hepatitis B e antigen (HBeAg) seroclearance between groups I and II.

median serum IFN-γ level was 14.8 pg/ml in group I and 15.3 pg/ml in group II, respectively (P=0.29). Following the treatment, a significant increase of the median serum IFN-γ level was identified in group II (20.9 pg/ml, P=0.0005) but not in group I (17.1 pg/ml, P=0.06), which was significantly higher in group II (P=0.004). The median serum TGF-β level was 672.9 pg/ml in group I and 630.5 pg/ml in group II prior to the treatment (P=0.15). Following the treatment, a significant reduction of the median TGF-β level was identified in groups I (338.9 pg/ml, P<0.0001) and II (286.0 pg/ml, P<0.0001), which was significantly lower in group II (P=0.002) (Table II).

Cumulative rate analysis. According to serum ALT detection, all the patients recruited exhibited a achieved biochemical response (4) after 48 weeks of therapy. A total of 24 patients in group I, and 27 patients in group II achieved SVR (11) during the treatment. SVR cumulative percentage rates at 6, 12, 24 and 48 weeks were 3.3, 20, 36.7 and 80% in group I and 23.3, 36.7, 66.7 and 90% in group II (P=0.0386). A total of 20 patients in group I, and 24 patients in group II achieved HBeAg seroclearance. HBeAg seroclearance cumulative percentage rates at 6, 12, 24 and 48 weeks were 3.3, 10, 20 and 66.7% in group I and 33.3, 50, 56.7 and 80% in group II (P=0.0442) (Fig. 1).

Discussion

Infection of hepatocytes by HBV appears to be non-cytopathic and the histopathology is a consequence of the adaptive

immune reaction to infection. During transient infections, which are generally <6 months in duration, Th1-type cytokines, including IFN-γ and TNF-β, potentially control HBV replication by activating virus-specific cytotoxic T cells, cluster of differentiation 8^+ ($CD8^+$) T-cell, natural killer (NK) cell and macrophage responses, consequently stimulating a cascade of inflammatory cytokines and degrading HBV RNA directly (12). In CHB patients, complete clearance of cccDNA cannot be spontaneously achieved due to inefficiencies in the innate and adaptive immune responses in these patients, in which high levels of Th2-type cytokines, such as TGF-β, may suppress innate antiviral immunity by blocking the cell cycle in G1, inhibiting the secretion of IFN-γ and TNF-α from HBV-specific T cells, impairing NK cell function by reducing NK cell receptor 2B4/SLAM-associated protein expression, and activating NK group 2 member D/DNAX protein 10 (13).

While nucleotide analogue monotherapy or with INF-α may not eliminate cccDNA directly, and treatment withdrawal is associated with reactivation of HBV replication, a number of other strategies utilizing host functions for HBV therapeutics are under way. For example, RNA interference, a major development in gene therapy leading to gene silencing, may be used to inhibit cccDNA amplification by targeting HBV nuclear localization signal, recruiting small interfering RNAs that are ~21 nucleotides in length and that hybridize to a homologous mRNA target, resulting in degradation of mRNA (14). Furthermore, by binding to the viral RNA substrate through its N-terminal zinc finger motifs, and in turn recruiting a host RNA processing complex, specifically the exosome, zinc finger proteins can markedly degrade HBV mRNA. An adoptive T cell therapy may also be a promising approach to ultimately eliminate cccDNA (15). While HBV-infected cells continuously produce HBsAg from the cccDNA template and a high number of hepatocytes (5-30%) remained positive for HBV S protein even following a long-term antiviral therapy, one modified cytolytic T cell carrying a chimeric T cell receptor may be designed to target S antigen-positive cells, and therefore license cytolytic T cells to eliminate these cells (16).

In general, current antiviral agents can control but not directly eradicate IH cccDNA. However, one previous study supports a direct deamination role of APOBEC3A (A3A) or APOBEC3B (A3B) on cccDNA, through interaction with the HBV core while sparing the cellular genome, and IFN-α and LT-β R agonists can result in extensive guanine to adenine (A) hypermutation and subsequent degradation of cccDNA mediating by A3A or A3B (17). Consequently, in combination with other antiviral strategies, the use of IFN-α and LT-β R agonists may be a potential to directly remove cccDNA from the nuclei of infected hepatocytes. Further studies are required to clarify the underlying mechanisms of this therapeutic strategy.

In China, *Cimicifuga* was firstly recorded to cure diseases in Shennong's Herbal Classic 2,000 years ago. Several types of evidence support the notion that the *C. foetida* extract is able to inhibit HBV replication, although the mechanism by which it does this remains to be established. However, two possible mechanisms can be postulated: i) *C. foetida* may interfere directly with the process of a HBV-DNA synthesisor; and ii) *C. foetida* may potentially induce cytokines or cell factors that enhance the degradation of HBV (18). While combination therapy has emerged as a new approach to the treatment of chronic HBV infection with the objective to decrease the occurrence of adverse effects relapse, the eradication of IH cccDNA during the combination therapy of ADV and *C. Foetida* was analyzed, and a significant reduction of the median IH cccDNA level was identified in group II but not in group I. The SVR and HBeAg seroclearance were significantly higher in group II compared to group I, indicating that combination therapy of *C. foetida* and ADV may be more superior to monotherapy of ADV in HBV transcription inhibition.

Of the specific inflammatory cytokines that are known to suppress HBV replication, IFN-γ, mainly produced by $CD4^+$ Th1 cells, $CD8^+$ T cells, NK cells, NKT cells (5), has a critical role in the suppression of HBV replication by stimulation of inducible nitric oxide (NO) synthase and production of NO, activating the nuclear factor-κB signaling pathway that destabilizes the integrity of HBV capsids, and degrades HBV mRNA by the formation of the inhibitor of κB kinase-α (IKK-α) and IKK-β (12,19). By contrast, TGF-β1, which was mainly released by $CD4^+$ $CD25^+$ cells, Th17 cells, Treg cells and $Foxp3^+$, may be the predominant immunosuppressive cytokines in HBV infection persistence. First, TGF-β1 may impair NK functions by reducing NKG2D/DAP10 and 2B4/SAP expression on NK cells. Second, TGF-β1 may suppress innate antiviral immunity by blocking the cell cycle in the G1 phase (20). In the present study, following the treatment there was a significant increase of the median serum IFN-γ level in group II but not in group I, and the median serum TGF-β level was significantly lower in group II compared to group I even when there was a significant reduction identified in the two groups. Therefore, we assume that *C. foetida* may be useful in the therapeutic management of HBV infection by stimulating IFN-γ production as well as inhibiting TGF-β secretion as a result.

In conclusion, this novel treatment suggests a new strategy for treating HBV infection. *C. foetida* may reduce the course of antiviral therapy, minimize the emergence of drug-resistant mutants, and reduce the financial burden of patients consequently.

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