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HD-03/ES: A promising herbal drug for HBV antiviral therapy

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ABSTRACT

Introduction: The present study was designed to study the genotypes associated with different groups of chronic liver disease and to see their response to HD-03/ES (an antiviral herbal molecule) on chronic HBV patients.
Methods: A total of 51 patients of chronic liver disease were recruited in the study and were given HD-03/ES, two capsules twice daily for 6 months. Liver function tests were done every month after initiating treatment. Serum was analyzed for HBsAg, HBeAg and HBV DNA and quantitative estimation of HBV at baseline, 4 and 6 months after therapy. The genotype of all the cases was also determined by PCR-RFLP method.
Results: After 6 months of therapy with HD-03/ES, a significant reduction of ALT values from 71.2 ± 16.3 to 36.4 ± 6.8 and a significant HBeAg loss (27.4%) and HBV DNA loss (27.4%) was observed. Adverse effects were mild. Genotype D was found in 39 (76.5%) while genotype A was found in 12 (33.5%) cases, respectively. The mean reduction in viral load was observed from $\log_{10} 7.1 \pm 1.8$ copies/ml to $\log_{10} 4.4 \pm 1.1$ copies/ml. However, a sharp decline in viral load was observed in patients infected with genotype A ($\log_{10} 6.8 \pm 2.5$ to $\log_{10} 4.9 \pm 1.8$; $P < 0.01$) compared to genotype D ($\log_{10} 7.0 \pm 2.6$ to $\log_{10} 5.9 \pm 3.5$; $P = 0.074$).
Conclusion: The study had shown that majority of the patients of chronic HBV related liver disease had genotype D. In additional, the molecule HD03/ES had a better therapeutic capability of lowering the HBV viral load in patients with genotype A, which needs to be validated in larger studies.

1. Introduction

Hepatitis B virus (HBV) infection is a major public health problem, with approximately 350 million individuals' chronically infected worldwide (Lee, 1997). HBV is highly endemic in sub-Saharan Africa, China and South-East Asia. It is also highly endemic in the Mediterranean basin and it is present at significant levels in most industrialized countries (Lee, 1997). As compared to Europe and North America, the prevalence of HBV infection in Asia is quite high, with 40 million people harboring chronic HBV infection in India (WHO). Two forms of chronic HBV infection can be individualized according to the presence or the absence of HBe antigen, but transitional forms exist (Ganem and Prince, 2004; Lee, 1997). Chronic HBV carriers are exposed to a risk of complications such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma, of which HBV is currently the most frequent cause (Ganem and Prince, 2004). Up to one million people die every year from the complications of HBV infection (Lee, 1997).

Prevalence of hepatitis B surface antigen (HBsAg) in India varies from 1 to 13 per cent, with an average of 4.7 per cent (Thyagarajan et al., 1996; Jain et al., 1992; Tandon et al., 1991). High prevalence rates of HBsAg have been noted among the Indian tribal population (Joshi et al., 1990; Jain et al., 1992; Murhekar et al., 2000). Various Indian studies have examined the proportion of persons with HBV infection among persons with chronic liver disease. Among patients diagnosed with chronic liver disease, the prevalence of HBsAg ranged from 33% to 75% (Acharya et al., 1993; Kant and Hall, 1995; Sarin, 1996; Sarin et al., 1988; Sundaram et al., 1990; Sundaravalli et al., 1988). Other series of patients with cirrhosis show HBsAg positivity ranging from 56% to 70% of cases (Dharmadhikari et al., 1990; Kant and Hall, 1995; Kelkar et al., 1975; Saxena et al., 1984; Sundaram et al., 1990). Histopathological studies of patients with liver cancer indicate evidence of HBV infection in 60–70% of cases (Kant and Hall, 1995).

Ayurveda, an indigenous system of medicine in India, has a long tradition of treating liver disorders with plant drugs (De et al., 1993). On the basis of leads available from folklore usage and recent experimental studies, HD-03/ES (a capsule formulation consisting of 125 mg each of hydroalcoholic extracts of the herbs Cyperus rotundus and Cyperus scariosus) was evolved to elicit hepatoprotective activity. Surface antigen suppression and HBV elimination

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activities of herbal extract containing Cyperus rotundus and Cyperus scariosus were examined using two HBsAg expressing human hepatocellular carcinoma cell lines, PLC/PRF/5 and HepG2.2.215 polymerase chain reaction (PCR) for the study of amplification of DNA specific to HBV, reverse transcriptase inhibition assay, immunomodulatory effects and hepatoprotective ability against oxidative damage to hepatocytes were some of the other studies performed to evaluate the efficacy of the plant extract (Yeh et al., 2003). The efficacy of the plant extract to eliminate the duck hepatitis B virus was assessed in experimentally infected Pekin ducks in a duck model study. An investigation indicated that the extracts could reversibly inhibit cell growth and suppress HBsAg expression in both of the human hepatocellular carcinoma cell line models (Yeh et al., 2003).

Acute and sub-acute toxicity studies conducted in rats indicated that HD-03/ES is devoid of significant toxicity following acute and repeated administration in rats (Mitra et al., 1998). However, a preliminary case study report of 25 chronic hepatitis B patients indicated that there was significant reduction of HBsAg along with the disappearance of viral DNA in a patient treated with HD-03/ES at a dosage of two capsules twice daily for a period of 6 months (Kulkarni et al., 2002). Another recent study by Rajkumar et al. (2007) also observed a significant HBsAg loss, HBeAg loss and HBV DNA loss after the period of 6 months therapy with HD-03/ES. However, there is no data available in north Indian patients to show whether HD-03/ES treatment is adequate for the treatment of HBV infection. We therefore, undertook this clinical study to evaluate the safety and efficacy of HD-03/ES in patients with chronic hepatitis B infection.

2. Materials and methods

2.1. Patients

An open labeled clinical trial of HD-03/ES capsules was carried out in 51 patients of chronic liver disease which included 41 (80.4%) patients of chronic hepatitis and 10 (19.6%) patients of decompensated cirrhosis who were admitted in the medical wards of Lok Nayak hospital and associated Maulana Azad Medical College, New Delhi, between June 2005 and May 2009 to evaluate the safety and efficacy of HD-03/ES capsules alone in the management of chronic hepatitis B infection. Informed written consent was obtained from all study participants and the protocol of the study was approved by the ethical committee of the institute. The study in general was conducted in accordance with the Declaration of Helsinki and GCP Guidelines issued by the Ministry of Health, Government of India.

2.2. Diagnostic criteria

Patients with the infection of HBV showing symptomatic, biochemical (alanine aminotransferase more than upper limit of normal) or serological (hepatitis B surface antigen (HBsAg), hepatitis B e-antigen (HBeAg), IgG antibodies to hepatitis B core antigen (IgG-anti-HBc) positivity), evidence of continued liver disease of more than 3–6 mo without steady improvement were diagnosed as suffering from chronic hepatitis B (CHB) (Ishak et al., 1995). The diagnosis of cirrhosis was established by clinical history or the presence of ascites and esophageal varices with small, irregular liver surface, altered echotexture and splenomegaly. HCC were diagnosed on the basis of either pathological or cytological examination or an elevated α-fetoprotein level (≥400 ng/ml) combined with atleast one positive image on Angiography, Sonography and/or Computerized Tomography.

2.3. Criteria for enrollment

Patients, aged 18–60 years, with their serum alanine aminotransferase (ALT) level being 41–200 IU/l and who had positive serum HBsAg, were enrolled.

2.4. Exclusion criteria

Patients aged over 60 years or less than 18 years, pregnant or lactating women, patients who had hepatitis C or other hepatic viral infection, autoimmune hepatitis and drug induced hepatitis or alcoholic hepatitis; patients with severe complications of the cardiovascular, renal or hematopoietic system; and patients with mental diseases, were excluded. Patients with a history of using interferon or antiviral agents or corticosteroids or immunosuppressive drugs were also excluded.

2.5. Treatment

Each patient was asked to take two capsules of HD-03/ES (The Himalaya Drug Company, Bangalore, India) twice daily, two capsules in the morning and two capsules at bedtime after food for a period of 6 months. The dosage proposed in this study was based on the dose escalation and safety studies carried out on human volunteers by the sponsors.

2.6. Recording and observation of symptoms and signs

The symptoms and signs of patients were recorded in detail using the "Clinical Observation Table" once a month before and during the treatment.

2.7. Liver function

The patients had liver function examinations every month during the treatment, including contents of serum proteins, total bilirubin (TB) and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

2.8. Etiological markers of hepatitis B

Serum samples collected from patients were stored at −20 °C until analysis. Serum was assayed for HBsAg, IgG anti HBc, hepatitis B e-antigen (HBeAg), and HBV DNA at baseline, 4 and 6 months after therapy using commercially available enzyme-linked immunosorbent assay kit.

2.9. HBV DNA detection (qualitative) from serum

DNA was extracted using phenol chloroform method. Briefly, 100 μl of serum sample diluted to 500 μl with reagent-grade distilled water. An equal volume of Tris–EDTA (TE)-saturated phenol (pH 8) was added, mixed thoroughly and incubated at 65 °C for 2 h. The tubes were centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatant was collected carefully and extracted once with an equal volume of TE-saturated phenol followed by an equal volume of chloroform and isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with two volume of ethanol and one-third volume of 7.5 M ammonium acetate at −20 °C for overnight, washed with 70% ethanol, air dried, and dissolved in 25 μl of Tris–EDTA (pH 8.0).

2.10. PCR amplification of HBV DNA

HBV DNA was detected by an in-house nested PCR technique, amplifying two different regions of the HBV genome as described

earlier by Datta et al. (2006). Amplification was performed in a total volume of 50 μl reaction mixture containing 5 μl of 10× buffer, 2.5 mM of dNTPs, 2.5 mM of $MgCl_2$, 50 pM of each primer, 2.5 units of Taq polymerase and 4 μl of DNA for 35 cycles. The amplified DNA product was electrophorized on 2% agarose gel stained with ethidium bromide at 150 V and analyzed with a U.V. transilluminator. Control samples included normal sera, HBV positive sera, and negative controls. The sensitivity of this PCR assay was also evaluated using the cloned HBV DNA for amplification which served as positive control. Positive and negative controls were run at HBV DNA extraction step as well as for PCR amplifications.

2.11. HBV DNA quantification by real time PCR

Single-tube assay with fluorescent hybridization probes and Light-Cycler Technology (Rotor Gene™ 2000, Corbett Research, Australia) was used to determine the HBV viral load. Briefly, the method consisted of a ready to use system for the detection and quantification of HBV using polymerase chain reaction (PCR) in the Rotor Gene 2000 (Corbett Research). The Specific Master Mix contains reagents and enzymes for the specific amplification of HBV and for the direct detection of the specific amplicons in fluorescence channel Cycling A.FAM of the Rotor Gene 2000 and the Reference gene on Cycling A. Joe. External positive standards (HBV S1–S5) with the specified concentrations are used to generate the standard curve which allows the determination of the gene load of unknown samples in subsequent runs. The PCR reaction was carried out as follows: initial hold at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 15 s. The lower limit of detection of this assay was 500 copies/ml with a linear range of up to 108 copies/ml. The HBV DNA values were $\log_{10}$ transformed for the analysis and reporting purposes.

2.12. PCR amplification of S-gene of HBV for genotyping

One microliter of extracted DNA was amplified by nested PCR in a 50 μl reaction mixture containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.75 U of Taq DNA (Bangalore Genei, Bangalore, India), 200 μM each dATP, dGTP, dTTP, dCTP and 10 pmol each of first-round sense primer (HBMF 1: 5′-YCCTGCTGGTGGCTCCAGTTC-3′) and anti-sense primers (HBMR 2: 5′-AAG CCANACARTGGGGGAAAGC-3′) and then the second round inner sense (HBMF 2: 5′-GTCT AGACTCGTGGTGGTGGACTTACTCTC-3′) and anti-sense primers (HBMR 2: 5′-AAGCCA NACARTGGGGGAAAGC-3′). After an initial 3 min denaturation at 94 °C, 40 cycles of amplification, including denaturation for 45 s at 94 °C; annealing for 60 s at 53 °C, and extension for 90 s at 72 °C (prolonged by 3 s/cycle) was done and followed by a final 7-min extension at 72 °C in a thermal cycler (MJ Research, USA). HBV S-gene sequence from nt 120 to nt 604 (485 bp) was amplified by the above primer sets.

Twelve microliter of the second round PCR product was electrophorized in 2% agarose gel at 70 V for 1 h and then visualized by U.V. fluorescence after staining with ethidium bromide. Band size of 485 bp was confirmed by comparing with known molecular weight marker (HaeIII-digested $\phi \times 174$ DNA).

2.13. Restriction digestion and RFLP analysis for HBV genotype identification

Restriction digestion was carried out with 15 μl of the second round PCR product for 3 h after adjustment with 10× enzyme reaction buffer according to the manufacturer's recommendations. Reactions would be carried out with 10 units of each of the following nuclease enzymes: AlwI, HphI, NciI, NlaIV and EarI (MBI Fermentas) at 37 °C. The digested PCR products were electrophoresed on 3.0% Nusieve GTG (3:1) agarose gel in 1× TBE buffer (134 mM, Tris–HCI, pH 10, 68 mM boric acid mM EDTA) containing 500 ng ethidium bromide per ml. The RFLP pattern was visualized under U.V. transilluminator as described by Mizokami et al. (1999).

HBV genotype B was distinguished by the fact that S-gene fragment remains uncut by EarI, while no AlwI site exists in S-gene sequences of genotype C. Only genotype E has a restriction site for NciI at position 461 and only genotype F has a restriction site for HphI at position 82. For genotype A, the specific restriction site for NlaIV is found at position 299, genotype D is digested at positions 265 and 299 by NlaIV.

Table 1
Demographic characteristics of the patients at the baseline.

Characteristics	$n = 51$
Age (years) (mean ± S.D.)	37.5 ± 7.8
Sex	
Males	38 (74.5%)
Females	13 (25.5%)
Body weight (kg)	59.2 ± 6.3

2.14. Safety analysis

Safety analysis included data for all treated patients during dosing. The primary safety end point was discontinuation of study medication because of adverse events. Other safety evaluations included incidence of adverse effects.

2.15. End points

The primary end point was HBsAg clearance. Secondary endpoints included HBV DNA levels and ALT normalization to 40 IU/l at the end of the treatment.

2.16. Statistical analysis

The intention-to-treat analysis included all randomized patients who were HBsAg-positive at baseline and received at least one dose of the study medication. Data were expressed as mean ± S.D. One-way ANOVA with Bonferroni's multiple comparison test or Dunnett's multiple comparison test was performed wherever appropriate using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA. A P-value of <0.05 was taken as statistically significant.

3. Results

Fifty-one patients (38 males and 13 females) aged between 19 and 51 years with a mean age of 35.9 years participated in this open study. Their baseline characteristics of the study subjects have been shown in Table 1.

3.1. Clinical response

Six months of therapy with HD-03/ES capsules was markedly effective in the majority of the patients as it resulted in disappearance or alleviation of chief clinical symptoms such as abdominal pain and poor appetite. The effect of 6 months of treatment with HD-03/ES on liver function tests is shown in Table 2 which shows a trend towards normalization of liver function tests in all patients treated with HD-03/ES. The levels of ALT were decreased from initial value of 71.2 ± 16.3 to 36.4 ± 6.8, and this reached levels of statistical significance ($P < 0.01$). In 32 of the 51 patients (62.7%),

Table 2
Response of HD-03/ES on the liver function profile of the subjects.

Parameters	Baseline	4th month	6th month
AST (IU/l)	51.6 ± 7.8	45.6 ± 5.9	42.3 ± 8.1
ALT (IU/l)	71.2 ± 16.3	46.2 ± 7.9	36.4 ± 6.8
Serum bilirubin (mg%)	1.3 ± 0.8	1.2 ± 0.6	1.1 ± 0.5
Alkaline phosphatase (IU/l)	159.7 ± 13.8	145.2 ± 10.6	131.1 ± 8.7
Total protein (g%)	6.3 ± 0.8	6.5 ± 0.9	6.6 ± 0.4
Serum albumin (g%)	3.5 ± 0.7	3.5 ± 0.5	3.6 ± 1.2
Serum globulin (g%)	2.9 ± 0.5	3.1 ± 0.4	3.2 ± 0.7

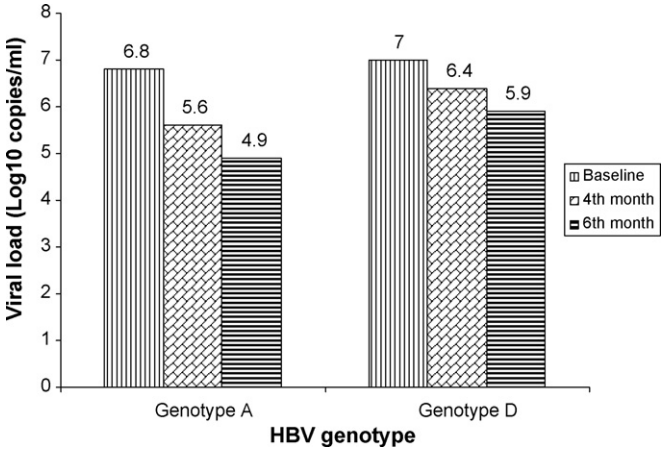

Fig. 1. Comparison of mean viral load among genotypes A and D infected patients.

ALT levels were normalized. Although ALT levels were not normalized in the remaining 19 patients, there was a trend towards reduction and in none of the patients was a rise in ALT levels seen.

3.2. Virological response

The effect of 6 months of treatment with HD-03/ES treatment on virological response is shown in Table 3. Six of the 51 patients (11.8%) at the end of treatment, who were treated with HD-03/ES, had undetectable HBsAg. Also HBeAg loss 14/51 (27.4%) and HBV DNA loss 14/51 (27.4%) observed during the treatment with HD-03/ES in patients who were positive for both HBeAg and HBV DNA initially. The mean baseline viral load of the 14 patients who cleared the HBV DNA was $\log_{10}$ 4.8 ± 0.79 copies /ml. Among these 14 patients only six patients cleared the virus at the end of 6 months. However, in the remaining six patients hepatitis B viral load decreases significantly at the end of 6 months, and subsequently cleared the virus after 12 months therapy with HD-03/ES. Genotype D was found in 39 (76.5%) while Genotype A was found in 12 (33.5%), respectively. The mean reduction in viral load was observed from $\log_{10}$ 7.1 ± 1.8 to $\log_{10}$ 4.4 ± 2.1 ($P < 0.01$). However, a sharp decline in viral load was observed in patients infected with genotype A ($\log_{10}$ 6.8 ± 2.5 to $\log_{10}$ 4.9 ± 1.8; $P < 0.01$) compared to genotype D ($\log_{10}$ 7.0 ± 2.6 to $\log_{10}$ 5.9 ± 3.5; $P = 0.074$) (Fig. 1).

3.3. Adverse events

HD-03/ES was well tolerated in this study. No patient was withdrawn from therapy either for adverse effects or for other reasons. Most of the observed side-effects were mild (fatigue, headache and insomnia) in nature. The most common adverse event was abdominal discomfort. No serious biochemical abnormalities were experienced by any patient.

4. Discussion

High morbidity and mortality have been found in Asia among HBsAg-positive patients, even in the absence of overt liver disease (Beasley, 1988; Sakuma et al., 1982). The goals of treatment in CHB infection are sustained viral suppression, normalization of ALT levels and improvement in liver histology leading to long-term reduction in the risk of cirrhosis and hepatocellular carcinoma (Jacobson, 2006). Loss of HBsAg, HBeAg and normalization of ALT levels and improvement in liver histology are the usual short-term end points of therapy (Yuen and Lai, 2003). The results of this study indicate that short-term therapy with HD-03/ES is effective in the management of CHB. The initial results of this study are promising, and hence we have extended the therapy in six responders which showed complete viral clearance including HBsAg, HBeAg and HBV DNA viral copies.

This primary observation on few responders to be very effective and hence it has to be seen in large number of cases whether virological response will be sustained during chronic dosing and whether relapse rates after cessation of therapy would be low unlike conventional therapies whose relapse rates are high after treatment cessation (Marcellin et al., 2004).

The ultimate endpoint of antiviral therapy for CHB infection is the loss of HBsAg, which is accompanied by disease remission in terms of ALT normalization (Flink et al., 2006). In this study, HBsAg loss was observed in 11.8% of the patients after 6 mo of therapy with HD-03/ES. This is in contrast to several clinical trials of lamivudine or Adefovir where HBsAg loss was not reported (Hadziyannis et al., 2000; Santantonio et al., 2000) or tends to occur later than 24 weeks as with interferon therapy (Brunetto et al., 2002). Although 6 months of therapy is limited and not capable of inducing pronounced viral suppression, six patients lost their HBV DNA after 6 months of therapy, which is highly encouraging. Loss of HBeAg either spontaneously or following therapy significantly improves the clinical outcome and survival in chronic HBV patients. Therefore, HBeAg loss has remained as a major end point of antiviral therapy in chronic HBV infection. Monotherapy with alpha interferon for 16–26 weeks is associated with the loss of serum HBeAg in 20–40% of the patients (Van Nunen et al., 2003). Our results (27%) are comparable to the interferon therapy. The HD-03/ES is also playing a promising role in lowering the viral load in almost all of the patients in our study. Further, we have also observed a sharp decline in viral load in patients infected with genotype A compared to genotype D.

The possible mechanisms of action as studied using HBsAg expressing human hepatocellular carcinoma cell lines PLC/PRF/5 and HepG2.2.2.15 indicate to HBsAg suppression by binding to the

Table 3
Presentation of viral factors at baseline and after 6 months of therapy.

Viral factors	Baseline		4th month		6th month	
	Positive	Negative	Positive	Negative	Positive	Negative
HBsAg	51	0	51	0 ()	45	6(11.8%)
HBeAg	51	0	45	6(11.8%)	37	14(27.4%)
HBV DNA	51	0	45	6(11.8%)	37	14(27.4%)
Mean viral load $\log_{10}$ (copies/ml)	7.1 ± 1.8		6.6 ± 0.8		4.4 ± 1.1	

antigen, and HBV elimination by reverse transcriptase inhibition. Immunomodulatory effects occur by causing the release of nitric oxide (NO) by macrophages and cytokines like TNF-α. It was found to have a hepatoprotective effect by reversing the oxidative damage caused by hepatocytes. A strong correlation was found between HBV DNA levels and histology activity index scores in HBeAg negative patients (Peng et al., 2003). The findings in the present study of ALT normalization, HBsAg loss together with loss of DNA during short-term treatment with HD-03/ES indicates that patients treated with HD-03/ES may lose their infectivity faster and relapse rates would be low.

Although the initial results of this study are promising, it remains to be seen whether virological response will be sustained during chronic dosing and whether relapse rates after cessation of therapy would be low unlike conventional therapies whose relapse rates are high after treatment cessation (Marcellin et al., 2003). Our study has several obvious limitations and among these we should consider the small sample size.

In summary, this trial demonstrated that 24 week of HD-03/ES treatment resulted in clinically significant virological and biochemical benefits in patients with CHB infection. Further, 6 months extended therapy gives comparatively better results in terms of viral clearance. Hence to conclude the potential benefit of HD-03/ES in the management of CHB, HD-03/ES should be studied in long-term comparative trials with standard drugs with extended duration of follow-up.

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