Original Article

Assessemnt of Biochemical, Serological, Molecular Viral Marker and Histological Parameters in HbeAg Positive Chronic Hepatitis B to Determine Therapy Response

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ABSTRACT

Introduction: About 300 million people worldwide have chronic hepatitis B virus (HBV) infection with varying degree of liver damage. The presence of continuing viral replication correlates with continuing disease activity and is associated with Hepatitis B e antigen (HBeAg) and hepatitis B virus DNA (HBV-DNA) in serum. Subsequently, the patient may undergo a spontaneous or therapy induced remission, which is accompanied by loss of HBV-DNA and HBeAg. This prospective study was undertaken to correlate all the above parameters so as to have an insight to monitoring of therapy in HBeAg positive chronic hepatitis B (CHB).

Aims and objectives: 1. To determine changes in biochemical, serological and virological profile in HBeAg positive CHB with therapy. 2. Determination of histology in liver biopsies in all cases and correlation with immunohistochemical detection of HBsAg and HBcAg with above parameters.

Methods: 42 patients of HBeAg positive CHB were enrolled and were followed up for 24 months. Blood samples were collected for alanine aminotransferase (ALT), hepatitis B surface antigen (HBsAg), Anti-hepatitis B core antigen (HBcAg), HBeAg and HBV-DNA. Liver biopsies were done in all individuals. Immunohistochemical staining for HBsAg and HBcAg were done where indicated. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software.

Results: There was a statistically significant improvement in the biochemical, serological and virological profile of the patients after therapy. However, the necroinflammatory activity showed improvement but was not statistically significant. Immunohistochemistry showed good correlation with viral load.

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Introduction

Hepatitis B Virus (HBV), a DNA virus accounts for more than 300 million cases of chronic infection and about 600,000 deaths each year worldwide and is a major health problem in Asia. [1,2] Progression to long term HBV infection occurs in approximately 15-40% of infected patients resulting in chronic hepatitis B (CHB) and depends on age, sex, immune status of the individual, viral load, replication of HBV and other factors. [3,4,5]

The natural course of CHB starts as Hepatitis B e Antigen (HBeAg) positive, immune tolerant phase which progresses to a HBeAg-positive immune-reactive phase, HBeAg-negative, inactive HBV carrier state, HBeAg-negative CHB phase and HBsAg negative phase (occult infection). [6,7] Histopathological changes include necroinflammatory activity and fibrosis, which are correlated with HBeAg, anti-HBe, alanine aminotransferase (ALT), and HBV DNA levels. [8] In CHB, HBeAg is an important marker of viral replication, infectivity and ongoing liver injury. Loss of HBeAg and acquisition of anti-HBe tends to be associated with biochemical and histological improvement**.** [9] During acute phase of infection, anti- hepatitis B core (HBc) of IgM class predominates. As the infection evolves, anti-HBc IgM levels gradually decline and often become undetectable within six months. [3, 9]

Antiviral therapy against HBV plays a pivotal role in determining the outcome of CHB as it can achieve control of viral replication, ALT normalization, HBeAg loss and seroconversion, and a small number of patients may achieve HBsAg seroconversion.[10,11] Numerous definitions have been used to assess response to antiviral therapy such as biochemical response (normalization of ALT); virological response (decrease in serum HBV DNA or loss of HBeAg with or without the development of anti-HBe); histological response (improvement in the histology activity index by at least 2 points without worsening of fibrosis score as compared to pretreatment biopsy); and complete response (biochemical and virological response with loss of HBsAg). [12,13]

Thus the aims and objectives of this study were to determine changes in ALT levels, serological and molecular viral marker profile along with histological parameters in HBeAg positive CHB to assess therapy response.

Material and Methods

In this prospective study, 42 cases of HBeAg positive CHB were followed for 24 months. Age and sex was no bar. History of any concomitant illness was taken into consideration but was not an exclusion criterion. Informed consent was taken and Institutional ethical clearance was obtained. They were treated with lamivudine with or without peg- interferon. They were evaluated for:

1. **Biochemical Parameters:** ALT were measured using ERBA kits in opERA system (BAYER) in accordance with principle based on International federation of clinical chemistry. method, kinetic. Quality control measures were strictly ensured.
2. **Serological Parameters:** HBsAg, HBeAg and IgM-anti HBc were performed by enzyme immunoassay (Milano, Italy). Positive and negative controls were run simultaneously to check the validity of test.
3. **Molecular Viral Marker:** Extraction of DNA was done on unhemolysed serum samples using AccuPrep Genomic DNA Extraction Kit by BIONEER which is a column-based assay. Quantitative PCR assays were carried out using HB V RG Real-ArtTM reagents in cycling A.FAM of the Rotor-Gene 2000 instrument. The detection occurs via the fluorescence labeling of oligonucleotide probes that bind specifically to the PCR amplicate and fluorescence intensity during the course of Real time PCR enables verification as well as quantification of the accumulating product. Samples with more than 10^5 copies/ml were considered positive.
4. **Histological Evaluation: S**pecimens were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin and sections cut to 3-4 um in thickness. Sections were subjected to hematoxylin and eosin stain and reticulin stain to study architecture; interface hepatitis; portal inflammation and lobular inflammation and fibrosis. Scores were accorded as per modified Knodell-Ishaak scoring system. [14] Immunohistochemical (IHC) staining was done in selected cases. IHC for HBsAg and HBcAg was done with monoclonal antibodies and ready to use kit manufactured by SEROTEC (USA).

Interpretation of IHC staining:

(a) HBsAg: strong brown staining of cytoplasm or membranous or both pattern of staining.

(b) HBcAg: strong brown staining of nucleus, cytoplasm or mixed pattern.

Statistical Analysis

The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD (Standard deviation). Wilcoxan assigned rank test was used to test the significance of two means. The level of significance "p" value was considered statistically significant if <0.05.

Results

All the 42 cases in this study were males with mean age of 34.5 (± 6.45 SD) years. Clinical symptoms of the patients were anorexia (73%) and nausea (70%), discomfort in hypochondrium and epigastrium (53%), weight loss (35%) and yellowish urine (27%).

1. **Biochemical Profile:** The mean value of ALT before treatment was 112.8 ±139.6 SD IU/L (Range= 26 to 696 IU/L) which reduced to 63.11 ± 62.4 SD IU/L (Range= 23-428 IU/L). Pre-treatment, 15 (35.7%) cases had normal ALT levels, 12 (28.6%) cases in subgroup 41-80, 08 (19%) in the subgroup 81-160 and 07 (16.7%) in the subgroup >160 IU/L. Post therapy, 23 (54.8%) cases had normal ALT levels, 08 (19%) were in the subgroup 41-80, 06 (14.3%) in the subgroup 81-160 and 05 (11.9%) in the subgroup of more than 160 IU/L. The decrease in ALT level was statistically significant (p value = 0.002).

   Summary of Pre- and Post-treatment ALT profile of HBeAg positive cases is shown in table no.1. Amongst these 16 cases (38.09%) had normalized, 05 (11.9%) remained static, 10 (23.8%) cases worsened whereas 11 (26.19%) cases improved but did not normalize.

2. **Serological Profile**: Before treatment, 39 (92.8%) were HBV-DNA positive and 03 (7.2%) cases were HBV-DNA negative. IgM anti-HBc was carried out in 32 cases, of which 12 (37.5%) cases were positive and 20 (62.5%) cases were negative. Out of 39 HBV-DNA positive cases, 23 (59%) became negative whereas 16 (41%) continued to remain positive. Of the 03 HBV-DNA negative cases, 01 case became positive whereas 02 cases continued to remain negative. Quantitative determination of HBV-DNA was done who were HBV-DNA positive, the mean viral load was 711,403, 098.35 -copies/ ml which reduced to the mean HBV-DNA level of 267,064,433.7-copies/ ml with treatment. Out of 12 IgM anti-HBc positive cases, 07 (58.3%) became negative whereas 05 (41.7%) cases remained positive. Of the 20 IgM anti-HBc negative cases, 02 (10%) became positive whereas 18 (90%) cases remained negative. HBsAg seroconversion was seen in 08 (19%) cases. All cases were HBeAg positive before therapy, post-treatment 35 (83.3%) became negative whereas 07 (16.7%) continued to remain HBeAg positive. Summary of Pre- and Post-treatment serological profile of HBeAg positive cases is as per table 2.

   Statistical correlation by Wilcoxan assigned ranks test shows statistically significant HBsAg seroconversion (Z value of – 4.38 and p value of 0.002), HBeAg seroconversion (Z value of – 5.96 and p value of 0.000), IgM anti-HBc seroconversion (Z value of – 4.45 and p value of 0.002) and HBV-DNA seroconversion (Z value of – 5.86 and p value of 0.000).

3. **Histological Profile**: Before therapy, 24 (57.1%) cases had KI score of less than 4, 10 (23.9%) were in the subgroup of KI score 5-8 and 08 (19%) in the subgroup of KI score more than 8. Post therapy liver biopsy was done in 15 cases of which 09 (60%) were in the subgroup less than 4, 04 (26.6%) in the subgroup 5-8, whereas 02 (13.4%) were in the subgroup more than 8. Pre-and post-treatment histological profile of HBeAg positive cases is shown in table 3.

   Four patients deteriorated histologically (i.e. > 2 points increase in activity). Of these, two patients were found to be was HIV positive. One patient did not show significant improvement or deterioration. Ten patients showed significant improvement (i.e. > 2 points improvement in histological activity). Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 1.605 and p value of 0.12.

Immunohistochemistry

1. HBsAg: Two patterns of staining were noted - Cytoplasmic and cytoplasmic + Membranous. Cytoplasmic positivity was seen in 20 (47.6%) of cases. The mean KI score in these cases was 3.8/22. Cytoplasmic and Membranous positivity was seen 16 (38.09%) cases with mean KI score of 6.2/ 22. This pattern was associated high titres of HBV-DNA (mean-633,807,112.75 copies/ml). Negative staining for HBsAg was seen in 6 (14.3%) cases.
2. HBcAg: Three patterns of staining are usually seen - nuclear, nuclear + cytoplasmic and only cytoplasmic. In this study following was observed:
   (a) Nuclear staining: This pattern of HBcAg staining was seen in 21 (50%) cases.
   (b) Nuclear and cytoplasmic pattern: This pattern of immunohistochemistry staining was observed in 18 (42.8%) cases. This pattern of staining was seen in cases with very high levels of HBV-DNA (mean=513,654,388.5 copies/ml). These cases had a mean KI score of 8/22.

   Only cytoplasmic pattern of staining was not seen.

Discussion

The presence of HBeAg in serum correlates with the presence of viral replication in the liver. It is recommended that detectable HBeAg should be taken as a surrogate marker for HBV DNA in hepatitis B virus carriers with raised serum ALT in case HBV PCR testing is not available. [8, 15]

Table 1: ALT Profile.

Pre-treatment ALT Profile (IU/L) n=42				Post-treatment ALT Profile (IU/L) n=42			
0-40	41-80	81-160	>160	0-40	41-80	81-160	>160
15	12	08	07	23	08	06	05

Table 2: Serological Profile.

HBeAg Positive cases (n=42)	Pre-Treatment Profile		Post-Treatment Profile	
			Remained Positive	Became Negative
HBV-DNA (n=42)	Positive	39	16	23
	Negative	03	01	02
IgM anti-HBc (n=32)	Positive	12	05	07
	Negative	20	02	18
HBsAg (n=42)	All cases positive		34	08
HBeAg (n=42)	All cases positive		07	35

Table 3: Histological Profile.

Pre-treatment Profile (n=42)			Post-treatment Profile (n=15)		
0-4	5-8	>8	0-4	5-8	>8
24	10	08	09	04	02

A. Biochemical Profile: ALT is not specific to hepatocytes and may be increased with injury to other organs; however, the most common cause of elevated ALT is liver disease. [16] In this study, 16 (38.1%) cases normalized and HBeAg seroconversion was seen in 35 (83.3%) cases. There was a significant decrease in mean post-treatment ALT levels (112.8 ±139.6 SD IU/L which reduced to 63.11 ± 62.4 SD IU/L).

However using serum ALT alone as marker for therapy response has limited value as patients have shown no improvement in necroinflammatory score in spite of achieving biochemical response.[17] In a recent study large scale study of CHB patients, significant number of patients who had persistently normal ALT levels (<40 IU/L) showed significant inflammation or fibrosis on biopsy.[18] The reason of this decreasing response rates with time can occur due to accumulation of YMDD mutants (Substitution of Isoleucine for Methionine at position 552) a virological breakthrough, which are always persistent.[19]

B. Serological Response: HBsAg seroconversion was seen in 08 (19%) cases. Serum HBsAg levels are known to reflect the presence of covalently closed circular DNA (cccDNA) in the hepatocytes and its clearance is thought to be the limiting factor for the elimination of infection.[20] Although, HBsAg seroconversion is most durable treatment endpoint it correlates poorly with therapy.[11] It occurs in 3-8 % of patients receiving interferon or peg-interferon and less than 2% of patients taking nucleoside analogues.[12] HBeAg seroconversion was seen in 35 (83.3%) out of 42 cases. HBeAg is used as an indicator of active underlying liver disease and high degree of infectivity. In contrast, the clearance of HBeAg from sera is associated with reduction in viral replication and normalization of transaminases.[21] Long term lamivudine therapy even after HBeAg seroconversion has shown additional benefit where relapse rate after stoppage of therapy was 13% at one year and 16% at two years, suggesting that long-term therapy might increase the durability of response.[22]

IgM anti-HBc is an indirect marker for acute phase of hepatitis and is the only marker for HBV detection in the "window period".[1,23] Out of 12 IgM anti-HBc positive cases, 07 (58.3%) became negative whereas 05 (41.7%) cases remained positive. Of the 20 IgM anti-HBc negative cases, 02 (10%) became positive whereas 18 (90%) cases remained negative. Thus, total 07 cases were positive after therapy. Out of these, liver biopsy was done in 02 cases, all of whom showed worsening of KI score compared to pre-treatment KI score. They were also found to be HIV positive. Semi-quantitative measurement of IgM anti-HBc has shown that antibody titer below 0.2 has 75% predictive of a mild necroinflammatory activity and rules out severe activity (29% sensitivity and 91.6% specificity) whereas antibody titer between 0.2 to 0.5 and more than 0.5 was associated with moderate and severe necroinflammatory activity, respectively. Although necroinflammatory activity correlates with IgM anti-

HBc levels, fibrosis was unrelated to IgM anti-HBc antibodies.[24] Quantitative IgM-anti HBc can be a novel biomarker for predicting treatment response in HBeAg-positive patients receiving therapy. [23]

C. **Molecular Viral Marker Profile:** HBV-DNA is the hallmark of active viral replication as it has been found in the liver biopsies of cases, which were HBsAg and HBeAg negative on serological examination. Molecular hybridization techniques have demonstrated HBV-DNA in liver biopsies in cases, which were anti-HBe positive and HBsAg negative. [25]

In this study, 23 (59%) cases became HBV-DNA negative whereas 16 (41%) cases remained HBV-DNA positive. Serial HBV DNA level profile can alter the course of therapy as studies suggest that initial viral kinetics during therapy can predict the sustained virological response in CHB. [11,15,26] Regarding cases, which became HBeAg negative but remained HBV-DNA positive are those which harbor mutations in the precore promoter i.e. A to G substitution at position 1896 in HBV genome.[27] Emergence of drug resistance conferred by mutations in the YMDD motif of HBV-DNA reverse transcriptase is a major problem in therapy. The prevalence of YMDD mutations increases with duration of antiviral therapy and has been detected in 20% of immunocompetent patients per year of treatment.[20,27] In our study, HBV-DNA positivity post-therapy is also probably due to emergence of mutant strains.

Histological Response

We used modified Knodell-Ishaak scoring system which includes interface hepatitis and bridging necrosis, lobular inflammation, portal inflammation and fibrosis. [14] In HBeAg positive and HBV DNA positive cases, 01 case remained static, 04 cases worsened while 10 cases improved. The improvement was seen in interface hepatitis, lobular inflammation, as well as in portal inflammation. Of the cases that improved, extent of fibrosis also improved in 07 (43.7%) cases (fig 1 A,B).

Amongst the cases, which worsened histologically, 03 cases continued to remain HBV-DNA positive after therapy whilst HBeAg seroconversion was seen in all cases. 02 of the cases, which worsened after treatment, were found to be HIV positive. Other case which worsened histologically may be case of some other chronic infection or reaction to drugs. The case, which became HBV-DNA negative with therapy but worsened histologically, might be harboring HBV-DNA mutants, which could not be detected during routine screening using conventional primers.

Immunohistochemistry Profile

We found that Cytoplasmic positivity for HBsAg (fig 2A) was present in 20 (47.6%) cases. Mean KI score in these cases was 3.8/22. Cytoplasmic and membranous pattern for HBsAg (fig 2B) was seen in 16 (38%) cases. Mean KI score in these cases was 6.2/22. These cases also had high viral load with mean HBV-DNA levels of 633,807,112.75 copies/ml. Negative staining for HBsAg was seen in 6 (14.3%) cases. Thus, overall 85.69 % cases were positive for HBsAg.

HBcAg profile was nuclear (fig 2C) in 21 out of 42 HBeAg positive cases. These cases had a mean KI score of 3.6/22. Nuclear and cytoplasmic pattern (fig 2D) was seen in 18 out of 26 HBeAg positive cases. These cases had a mean HBV-DNA level of 513,654,388.5 copies/ml and a mean KI score of 8/22. 03 HBeAg positive cases did not show positive staining for HBcAg. The reason for negative can be explained on the basis of sequencing analysis of integrated viral DNA which suggested that the HBsAg gene remains intact whereas the HBcAg gene gets either deleted or rearranged resulting in impaired synthesis of HBcAg in the liver with integrated HBV-DNA. [28]

Significant correlation has been found between intrahepatic HBcAg expression with HBeAg and HBV-DNA ($p<0.001$) with highest levels of HBV-DNA found in the cases with nuclear and cytoplasmic pattern of staining (mean= 10^6 viral genomes/ml). Significant link between HBV-DNA and membranous pattern of HBsAg staining has also been found ($p=0.001$).[29] According to pathogenetic theory the immune response to HBeAg (membrane bound nucleocapsid antigen) is responsible for liver damage, while the immune response to free HBcAg has no apparent antiviral effect since the nucleocapsid is always masked within the HBsAg envelope of the virion.[30] Furthermore, the data suggests that HBcAg expression is not associated with integrated form of HBV-DNA and HBsAg staining can be seen in integrated as well as episomal forms of HBV-DNA. [28]

Conclusion

Ours was a prospective study, which evaluated 42 cases of HBeAg positive CHB cases. After treatment, we found statistically significant decrease in mean ALT levels and statistically significant HBsAg, HBeAg, IgM anti-HBc and HBV-DNA seroconversion. Improvement was observed in histological profile but was not statistically significant. Based on these findings we conclude that single most reliable marker for assessing therapy-induced response is HBV-DNA. Persistence of IgM anti-HBc post

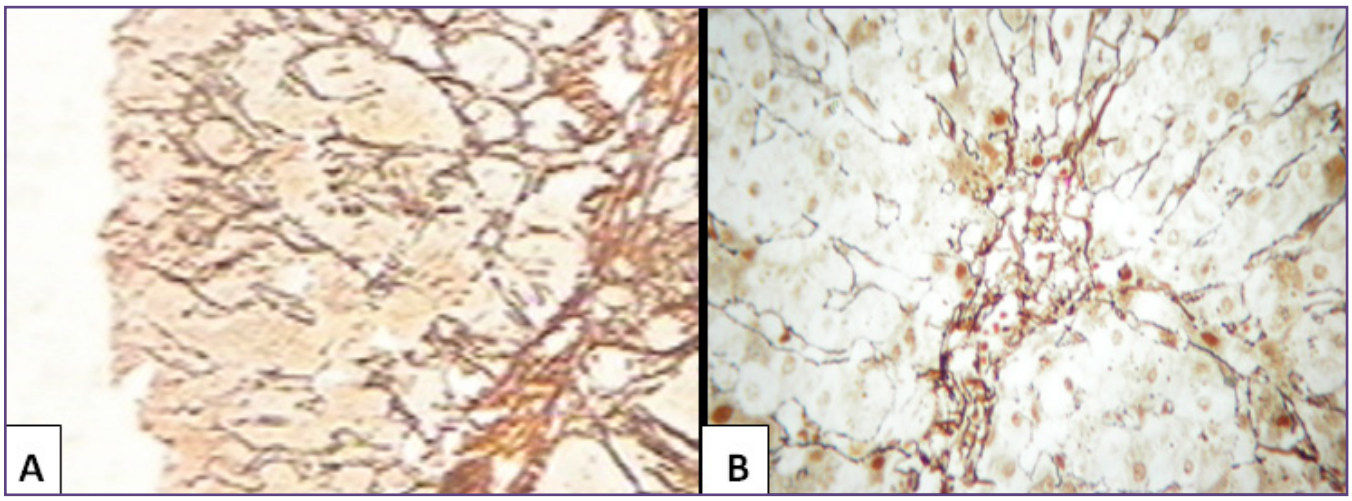

Fig. 1: Improvement in fibrosis (A-pre-treatment; B-post treatment) with therapy (Reticulin stain, 20x).

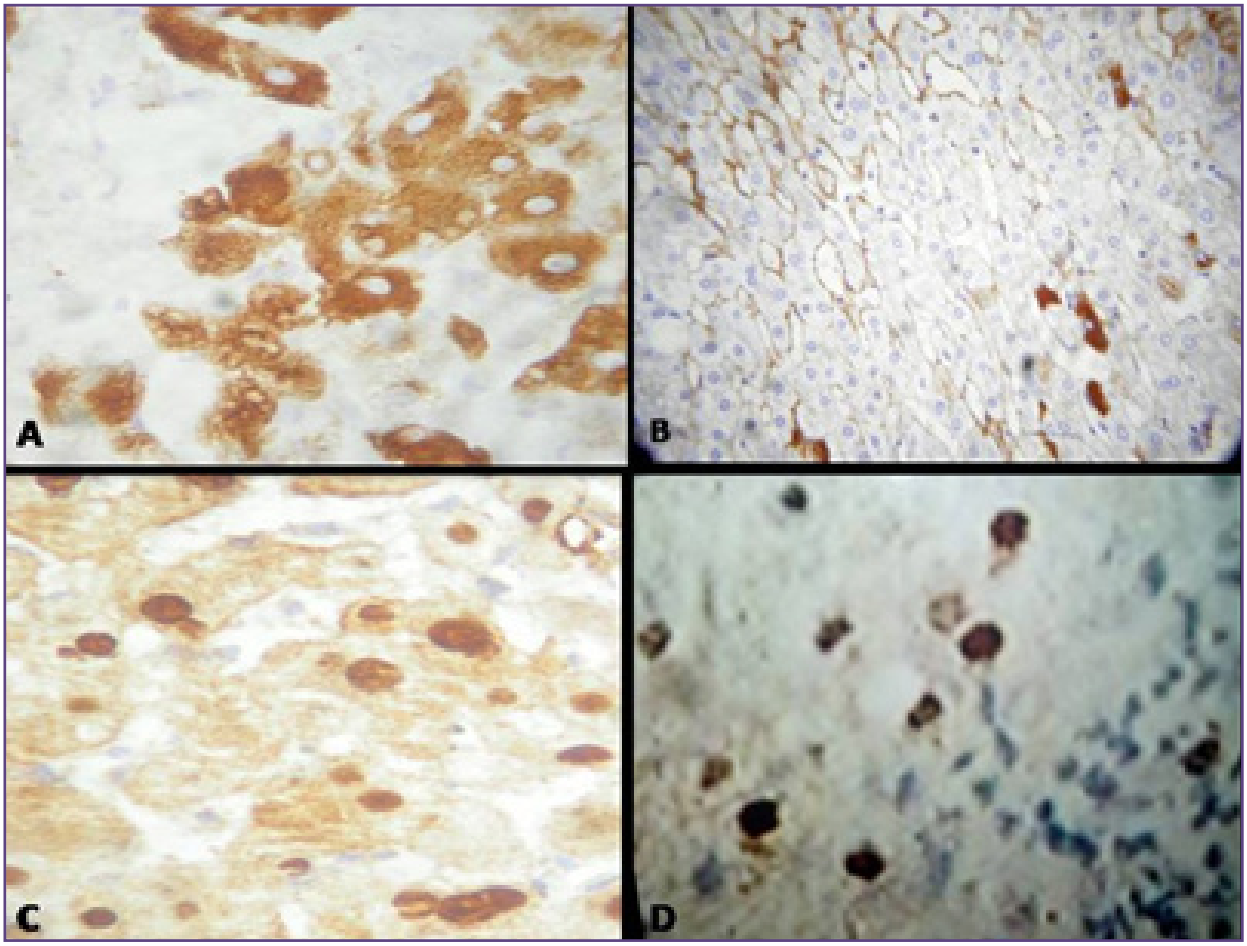

Fig. 2: Cytoplasmic (A) and cytoplasmic + membranous (B) pattern of HBsAg immunohistochemistry staining. Nuclear + cytoplasmic (C) and nuclear (D) pattern of HBcAg immunohistochemistry staining.(Immunohistochemistry stain, 20x).

therapy denotes continuing necroinflammatory activity. Histological profile did show improvement but was not statistically significant in our study. In IHC, nuclear and cytoplasmic pattern of HBcAg staining is associated with high levels of viremia and marked necroinflammatory activity.

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Conflict of Interests
All authors have none to declare

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