ORIGINAL ARTICLE

Impact of the Route and Schedule of Immunization on the Serological and Virological Response of Chronic Hepatitis B Patients Treated with HeberNasvac

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Abstract

HeberNasvac is a recently developed therapeutic vaccine for chronic hepatitis B (CHB) administered by intranasal (IN) and subcutaneous (SC) routes in a 14 days/10 doses schedule. To compare different schedules and routes of immunizations, a group of patients received four different vaccination regimens in a placebo-controlled factorial study. Subsequently, patients were followed for a minimum time of 48 weeks. Samples collected at the end of the follow-up were compared with initial samples. Groups I and II received the product by IN/SC routes, every 14 and 7 days, respectively. Groups III and IV were treated by SC route alone following a 14 and 7 days schedule.

A group of 21 CHB patients received the vaccine in four different schedules and eight patients received placebo for a total of 29 patients enrolled. The 61.9% of vaccinees reduced their VL ≥2Log compared with baseline levels and 25% in placebo group. The 47.6% of vaccines reduced HBV levels to undetectable, 25% in placebo. HBeAg loss and seroconversion to anti-HBeAg was only achieved in vaccinees, 4 out of 9 (44.4%), and 40% (8 out of 20) developed anti-HBs response, none in placebo group. Reduction of HBsAg level in ≥1Log was achieved in the 35.0% of vaccinees and in none of the placebo-treated patients. Considering the individual and factorial analysis, significant HBV DNA reduction was detected in groups I and II, immunized by IN/SC routes. A significantly higher proportion of patients reducing VL to ≥2Log was also detected grouping the patients treated by IN/SC routes (G I + II) and grouping those inoculated every 14 days (G I + III), with 72.7% and 63.6%, respectively, compared with the placebo group (25.0%). The patients immunized every 14 days (G I + G III) also reduced the HBsAg levels compared with baseline.

In conclusion, after more than 48 weeks of treatment-free follow-up, HeberNasvac-treated patients demonstrated superior responses compared with the placebo group in terms of antiviral and serological responses. The factorial analysis evidenced that the schedule combining the IN route of immunization and the frequency of 14 days resulted in the stronger antiviral and serological responses. Present results support the study of IN-only immunization schedules in future and was consistent with previous results. Long-lasting follow-ups were done to explore histological variables and the progression of serological variables in order to detect late responders.

Keywords: Chronic hepatitis B, HeberNasvac, Hepatitis B Virus, Hepatitis B surface antigen, Therapeutic vaccines.

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Introduction

Chronic hepatitis B (CHB) is a disease affecting around 350 million persons worldwide.[1] Chronic HBV carriers are susceptible to develop hepatocellular carcinoma (HCC), cirrhosis, and other complications that result in half a million deaths per year.[2,3] Approved therapies for CHB are Interferon alpha (IFN-α), alone or conjugated to polyethylene glycol (PEG-IFN-α) as well as nucleos(t)ide analogs like Entecavir and Tenofovir (NUCs). The prolonged duration of the treatment with NUCs and the adverse events related to PegIFN treatment, linked to poor efficacy in terms of HBsAg elimination limit their effectiveness.[4–8]

Therapeutic vaccination remains as a valid approach as immune therapy as the ultimate goal of HBC treatment is the disappearance of HBsAg with the development of antibodies against the hepatitis B surface antigen (anti-HBs), which is associated with a favorable clinical outcome[9–11] and in this sense therapeutic vaccination offer several advantages.[12–15]

HeberNasvac is a therapeutic vaccine, produced and commercialized by the Center for Genetic Engineering and Biotechnology (CIGB), in Havana, Cuba. This formulation comprises the recombinant HBV surface and core (HBsAg and HBcAg) antigens, which has demonstrated excellent immunogenic properties and safety profile

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Conflict of interest: Dr Eduardo Penton, Dr Mamun Al Mahtab, Dr Sheikh Mohammad Fazle Akbar, and Dr Julio Cesar Aguilar are

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in the preclinical[16–18] and clinical trials.[19–26] The recombinant HBV nucleocapsid antigen (HBcAg) is used both as a potent Th1 response adjuvant for the coadministered HBsAg, as well as a relevant vaccine antigen.[27,28]

In this work, variables as viral load (VL), HBeAg to anti-HBeAg seroconversion as well as quantitative HBsAg and anti-HBsAg levels were evaluated in CHB patients vaccinated with HeberNasvac, comparing two alternative immunization schedules (subcutaneous and intranasal vs subcutaneous alone) and two different intervals inter-immunizations (every 14 days and every 7 days) in order to detect potential alternatives to optimize the schedule of immunization that is currently in use with this product.

Materials and Methods

HeberNasvac and Placebo Formulations

The therapeutic hepatitis B Vaccine, currently registered under the trade name of HeberNasvac®, was produced and provided by the manufacturer (CIGB, Havana, Cuba). Each dose contains 1 mL of HeberNasvac, in two presentations (IN, 6R vials) and SC (2R vials). HeberNasvac comprises 100 µg of each, HBsAg and HBcAg antigens in phosphate-saline buffer. Placebo formulation only contains the buffer excipients, a saline solution with phosphates, NaCl and EDTA, pH7. No preservants, adjuvants or any additional additives are included in neither the vaccine nor the placebo.

Patients

A total of 29 HBsAg (+) CHB patients, of both sexes, with HBV levels ≥10^4 copies/mL, and without other treatment for more than 6 months, were recruited to participate in a phase II clinical study, of factorial design, randomized, placebo-controlled and double-blind, conducted by the Department of Clinical Trials of the Center for Genetic Engineering and Biotechnology of Havana, Cuba (CIGB). The study received the approval of the Ethical Committee from each of the six hospitals involved in the trial, and the authorization of the Cuban Center for the State Control of Medicaments (CECMED).

Study Groups and Blinding Procedure

Patients were randomly allocated into four treatment groups (TG) and 1 placebo control group (PG). The samples size per group after decoding resulted in $N = 6$ (G I), $N = 5$ (G II-IV) and $N = 8$ (G V). G I and G II received a first cycle of 5 and 10 doses by the IN route, every 14 and 7 days, respectively, and a second cycle of 5 and 10 simultaneous IN/SC administrations, for a total of 10 and 20 immunizations, respectively. Patients assigned to groups III and IV received 5 and 10 doses of HeberNasvac, at intervals of 14 and 7 days by SC route, in cycle 2 only. The placebo was administered in G5 following the schedule of G2 and also to the rest of the groups to complete a G2-like schedule of treatment, in order to ensure the blinding.

Samples

Serum samples were collected at the hospitals and transferred as blind samples to the Analytical Laboratory of Biomedical Research Direction, at CIGB, and preserved at –80°C until evaluation. The studied samples were taken prior to the beginning of vaccination (week 0) and after a minimum follow-up period of 48 weeks, corresponding to the last extraction. Samples were taken according to the double-blind procedure, and analyzed by blind analysts.

associated as the Editorial board members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial board members and their research group.

Consent for publishing: Publication of these results has been approved by all coauthors and our institutional authorities.

Serum HBsAg Quantification

An in-house quantitative system was validated at CIGB, briefly, the wells of the microelisa plate (COSTAR, Cat. No. 3590) were coated with a mixture of two monoclonal anti-HBs antibodies (adw2) (CBHep.1 and CBHepB4, CIGB *Sancti spiritus*, Cuba). After addition of the test sample or appropriate control containing HBsAg, incubation for 1 hour at 50°C was completed. Three washes were carried out with a distilled water solution containing 0.05% Tween-20. Then the horseradish peroxidase-labeled anti-HBs conjugate (HRP-CBSSHepB.4, CIGB *Sancti spiritus*, Cuba) was added. The labeled antibody binds to the solid phase HBsAg/anti-HBs complex. After washing and incubating with the OPD/H_2O_2 substrate, the reaction was stopped with sulfuric acid 3M stop solution. Samples with OD value >2X the OD of the negative control were positives. A standard curve was created with a reference material at different concentrations to allow quantification. The sensitivity was established at 6 ng/mL.

HBsAg Qualitative Detection

HBsAg presence/loss was assessed using the commercial ELISA (DIA source, Belgium). The analytical sensitivity of this assay was 0.03 IU/mL (WHO's HBsAg secondary international standard), subtypes *adw2*, genotype A, NIBSC code: 00/588 Cat.No KAPG4SGE3/ KAPG4SGE11, Belgium).

HBV Quantitative Determination

Viral DNA purification was performed using the reagents and procedures previously validated at the CIGB (Habana, Cuba).[29] Viral DNA values below 250 copies/mL were considered as non-detectable.

HBeAg/anti-HBeAb Seroconversion

HBeAg/anti-HBeAb were detected using the commercial DIAsource reagent set (Cat. # KAPG4BN3, Belgium), following the manufacturer's instructions.

Statistical Analysis

For data tabulation and statistical analysis, Excel and GraphPad Prism (version 6, USA) softwares were used. The exploratory analysis was carried out by treatment groups and also considering the factorial analysis, combining administration routes and treatment schedules according to study design. A logarithmic transformation was applied for the analysis of the HBV DNA and qHBsAg variables. Continuous and categorical variables are expressed as means ± standard deviation and proportions (percent), respectively. To assess the mean change at the end of follow-up vs baseline values, a paired Student's *t*-test was applied when the distribution of values accomplish normality and homoscedasticity or the Wilcoxon signed rank test, if not fulfilled. To compare means of unpaired samples, the *t*-test (normality and homoscedasticity fulfilled) or the Mann–Whitney test, alternatively. For the comparison of proportions, the Chi-square (χ^2) test was used. The value of $p = 0.05$ was considered a significant difference. All statistical tests were considered two-tailed.

Table 1: Virological and serological characteristics *per* group. HBV DNA [Log(copies/mL)] and HBsAg [Log(ng/mL)] levels are analyzed quantitatively and qualitatively before the immunization [Baseline (W0)] vs >48 weeks after the end of treatment (F-up)

Variables	*Group I N = 6*	*Group II N = 5*	*Group III N = 5*[†]	*Group IV N = 5*	*Group V N = 8*	*Total (N = 29)*
HBV DNA levels at W0 (Mean ± SD)	6.4 ± 1.2	5.7 ± 2.5	5.7 ± 2.2	5.6 ± 1.1	5.7 ± 1.3	6.1 ± 1.3
HBV DNA at F-up (Mean ± SD)	4.1 ± 1.9	3.7 ± 1.5	3.5 ± 2.1	4.3 ± 2.1	4.9 ± 1.9	4.3 ± 1.9
Mean change (*p*-value)[†]	0.023	0.03	0.063	0.254	0.083	
Reduction ≥2log (*n*, %) at F-up	4 (66.7)	4 (80.0)	3 (60.0)	2 (40.0)	2 (25.0)	15
Reduction <250 c/mL (*n*,%) at F-up	3 (50.0)	2 (40.0)	3 (60.0)	2 (40.0)	2 (25.0)	12
HBeAg (–) serology at W0	2	2	4	3	6	17
HBeAg (+) serology at W0	4	3	0	2	2	11
HBeAg to HBeAb (*n*, %)	2 (50.0)	2 (66.7)	0	0 (0,0)	0 (0.0)	4
HBsAg [Log(ng/mL)] at W0 (Mean ± SD)	3.3 ± 0.5	2.3 ± 1.5	2.2 ± 1.1	3.3 ± 1.5	3.2 ± 0.9	3.0 ± 1.0
HBsAg [Log(ng/mL)] F-up (Mean ± SD)	3.0 ± 0.7	1.6 ± 1.0	1.8 ± 0.8	2.5 ± 1.9	2.9 ± 1.1	2.5 ± 1.2
Reduction ≥1log (*n*, %)	1 (16.7)	2 (40.0)	2 (50.0)	2 (40.0)	0 (0.0)	7
HBsAg loss (n, %)[‡]	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	1
HBsAb detection (*n*, %)	3 (50.0)	1 (20.0)	2 (50.0)	2 (40.0)	0 (0.0)	8

SD, standard deviation. [†]Mean change comparison *p*-value: Statistical significance $p \leq 0.05$, paired *t*-test comparison Baseline (W0) vs Follow-up (F-up); [‡]Only four patients studied for serology

Results

Baseline Virological and Serological Characteristics of the Study Population

Samples from a total of 29 HBsAg-positive patients, with levels of HBV ≥10^4 copies/mL were available for the virology study and 28 for the study of serological variables. At the beginning of the study, 17 patients (60.7%) were HBeAg(–) (Table 1). The mean HBV DNA levels of the whole population of patients was (6.1±1.3) Log[copies/mL]. In HBeAg(+) patients, the initial HBV DNA level was 6.7 ± 0.8 while in HBeAg(–) was 5.6 ± 1.3 Log[copies/mL] ($p = 0.012$, Mann–Whitney, two-tailed). The mean qHBsAg level at the beginning of the study was 3.0 ± 1.1 Log [ng/mL]. Although no significant differences were detected between HBeAg(+) and HBeAg(–) patients (3.3 ± 1.1 vs 2.8 ± 1.1 Log[ng/mL], $p = 0.323$, Unpaired *t*-test), respectively (Table 1), the mean qHBsAg value favored the HBeAg(+) patients (Table 1).

Considering the baseline virological and serological characteristics between treated and non-treated groups, statistically similar levels of HBV DNA were detected between groups (6.3 ± 0.3 vs 5.7 ± 0.4 Log[cop/mL], $p = 0.24$, Unpaired *t*-test). Similarly, qHBsAg levels were not different between treated and non-treated groups at baseline (3.3 ± 0.2 vs 3.0 ± 0.3 Log [ng/mL], $p = 0.41$, Unpaired *t*-test).

Virological and Serological Responses in Treated and Non-treated Patients

The mean change of HBV DNA (preimmune vs end of follow-up) between the treated and non-treated patients evidenced a significant reduction only in the treated group, but not in untreated ($p = 0.0007$ vs $p = 0.082$; paired *t*-test). Similar results were detected for qHBsAg ($p = 0.003$ vs $p = 0.088$; Paired *t*-test).

Taken together, a 41.2% of the treated patients reduced the qHBsAg levels in >1 Log (considering the patients with detectable HBsAg values at baseline), while 1(8) patients (12.5%) reduced the qHBsAg levels >1 Log in the non-treated group ($p = 0.15$, χ^2). Only vaccinated patients resulted in undetectable qHBsAg values at the end of the follow-up [7(20); 35%; $p = 0.05$, χ^2]. In the case of HBV DNA, vaccinated patients resulted in superior (but non-significant) virological response with 10(21), 47.6% patients with undetectable levels of HBV DNA at the end of the follow-up vs 2(8), 25% in the non-treated group ($p = 0.27$, χ^2).

The antibodies against HBsAg (Anti-HBsAg serological response) was only detected in treated patients. Patients with anti-HBsAg serology at the end of the follow-up evidenced a higher drop of qHBsAg values compared with those seronegative for anti-HBsAg ($p = 0.03$, Paired *t*-test, mean change 1.12 Log [ng/mL]). A drop in qHBsAg levels was also detected in anti-HBsAg seronegative patients, although not significant ($p = 0.11$, Paired *t*-test, mean change 0.62 Log[ng/mL]). The impact of anti-HBsAg response in the drop of the HBV DNA was not detected. In both subgroups – with and without anti-HBsAg response – the HBV DNA evidenced a very significant reduction ($p = 0.001$ and $p = 0.006$, respectively).

The sample of HBeAg(+) patients in the present study was reduced to 11 out of 29 patients involved in the analysis. A total of 9 were treated and 2 remained untreated. Seroconversion of HBeAg was achieved in 4 out of 9 vaccine-treated patients (44.4%). None of the 2 HBeAg (+) in the untreated group seroconverted.

Virological and Serological Response according to Treatment Group

In groups I and II, a significant reduction in VL was achieved after 48 weeks follow-up period compared with baseline ($p = 0.023$ and $p = 0.021$, respectively, Paired *t*-test). There was no significant reduction in the mean HBV DNA levels in the rest of the groups (Table 2). In general, the patients treated with HeberNasvac (GI–IV) showed a superior frequency of reduction below 2Log the baseline values (66.7%; 80%; 60% and 40%) compared with the patients in the placebo group (25%). In group V (Placebo), the VL was reduced in 2 out of 8 (25%) of patients and in both patients the reduction occurred to undetectable levels (Table 2).

No significant differences were detected after studying the changes in the qHBsAg mean values between the end of the follow-up and the baseline levels per group. A total of 4 out of 9 patients seroconverted to anti-HBeAg with HBeAg loss. HBeAg responses were detected in vaccine recipients only. Specifically, HBeAg seroconversion was detected in patients immunized by IN/SC routes (G I or G II) (Table 1).

Table 2: Proportion of patients that reduced HBV DNA and HBsAg levels, according to the factorial analysis by routes and immunization schedules

	Routes and Schedules of administration				
Reduced variable (Response)	*G I + II (IN/SC) N = 11*	*G III + IV (SC) N = 10*	*G I + III (e/14 d) N = 11*	*G II + IV (e/7 d) N = 10*	*G V (placebo) N = 8*
VL ≥2Log	8 (72.7)	5 (50.0)	7 (63.6)	6 (60.0)	2 (25.0)
p-value (*W0* vs *FU*)	0.002	0.02	0.002	0.011	0.083
p-value (*vs* G5)[†]	0.040	0.280	0.096	0.138	–
HBsAg ≥ 1Log	3 (27.3)	4 (44.4)[‡]	3 (30.0)[§]	4 (40.0)	0 (0.0)
p-value (*W0* vs *FU*)	0.040	0.047	0.004	0.062	0.089

Comparison against Group V (χ^2-test). [†]Reduction of VL to ≥2Log and HBsAg level to ≥1Log the baseline values; [‡]In this group, nine samples were analyzed for HBsAg; [§]In this group, 10 samples were analyzed for HBsAg

Virological Responses Combining Groups according to Factors

The analysis of the HBV DNA levels considering all the combinations of factors detected a significant increase in the proportion of patients with HBV DNA drops >2Logs for all combinations (Table 2); however, the combination of G I + G II (patients immunized by IN/SC routes) and G I + G III (immunized every 14 days) were the groups with a very significant drops in their viral loads compared with preimmune (W0) values (Table 2).

The proportion of patients with HBV DNA reduction ≥2Log was higher in groups receiving the vaccine by IN/SC routes (G I + G II), compared with the patients treated by SC route alone (G III + G IV), although a non-significant difference was found (72.7% vs 50.0%, respectively, $p = 0.284$, χ^2). A significant difference was detected when the IN/SC groups (G I + G II) was compared with placebo ($p = 0.04$, χ^2), not in the case of G III + IV vs G V ($p = 0.280$, χ^2), see Table 2.

Serological Responses Combining Groups according to Factors

In factorial groups G I + G II and G III + G IV, the HBsAg level decreased significantly in relation to the initial level ($p = 0.040$ and $p = 0.047$, paired *t*-test). On the second factorial analysis considering the frequency of administration (every 7 or 14 days), a significant reduction in HBsAg level was achieved in the group G I + G III (every 14 days), compared with the baseline values ($p = 0.004$).

Discussion

The final goal of chronic hepatitis B treatment is to avoid the development of liver cirrhosis, HCC, and ultimately death related to hepatitis B infection. Current therapies with nucleotide analogues effectively suppress viral replication and normalize transaminases on treatment;[9–11] however, this effect is frequently a loss off-therapy. In the present study, we are evidencing that HeberNasvac-treated patients, followed by a minimum of 48 weeks after the end of immunizations, remain with a low viral load in the majority of the patients, with almost half of the patients with still undetectable viral loads. Even when such results were obtained with different immunization routes and schedules, they are consistent with the results of the phase III clinical trial previously reported[23–25] where vaccinated patients evidenced a long-lasting virological suppression.

It is important to highlight that 41% of vaccinated patients reduced the level of qHBsAg in >1Log [ng/mL], most of them (35%, 7(20)) were undetectable, while none of the non-treated patients resulted in qHBsAg undetectable by the same system. The drop in HBsAg was more pronounced in the patients developing detectable anti-HBsAg response, a result showing consistency between the variables. Regarding HBeAg, 4 out of 9 vaccinated patients with positive serology at the beginning of the study loss the HBeAg and seroconverted to anti-HBeAg. Although the sample size was reduced, these are relevant results considering that the elimination of serum antigens (HBeAg and HBsAg) and the seroconversion to anti-HBeAg and anti-HBsAg limited the achievement of current treatments. Interferon-based treatments achieve 20–30% HBeAg seroconversion and 5–10% of HBsAg loss depending on the specific genotypes and other conditions and timings. NUCs impact on these variables is even poorer.[9–11]

The analysis of the virological response by individual groups highlighted the relevance of the IN route of immunization, as only the IN-treated groups reduced the viral load significantly comparedwith the baseline values and the frequency of patients with >2Log drop in the HBV DNA was higher in IN immunized groups. In line with this, only IN immunized patients succeed in HBeAg/anti-HBeAg seroconversion, nor in SC immunized groups neither in the non-treated control group. These results are consistent with the reported capacity of the IN route alone or in combination with the SC route, to induce antiviral therapeutic immune responses as well as preferential immune responses in the liver[21–26] as well as with studies showing the impact of the IN route after the first cycle of treatment.[21–26] Taken together, these studies further support the effect of the IN immunization, alone, or combined with the SC route.

The analysis of the factors grouping the patients by similar routes or frequency of immunizations further confirmed the results of the individual analysis regarding the impact of nasal administration; however, it was possible to find that the patients immunized every 14 days (G I + G III) also reduced the viral load compared with their baseline levels as well as the qHBsAg levels compared with baseline, but not in the more intense vaccination approach every 7 days that represents the double number of immunizations and thus the double total dosage. These results further confirm the value of the 14 days immunization schedule[29,30] that is being used in the clinical practice with HeberNasvac.[24]

The present study requires further optimization steps, mostly to explore extended treatment regimens or additional cycles of immunization in order to optimize the elimination of the HBsAg/HBeAg and their seroconversion to more competitive levels. We also consider additional studies in larger HBeAg (+) populations as the precedent results[24–26] are consistent with the present data in terms of strong HBeAg seroconversion and this is a relatively rapid efficacy variable compared with HBsAg. Others products such as NUCs and IFN, report HBeAg loss between 10 and 30%, respectively[9–11] Long-lasting evaluations are warranted in order to explore the progression of the serological and virological responses after 2, 5, and 10 years of HeberNasvac immunization.

In conclusion, after more than 48 weeks of treatment-free follow-up, HeberNasvac-treated patients demonstrated superior

responses compared with the placebo group in terms of antiviral and serological responses. The factorial analysis evidenced that the schedule combining the IN route of immunization and the immunization schedule of 14 days resulted in the stronger antiviral and serological responses. These results support the use of IN-only immunization schedules with HeberNasvac and the development of stronger formulations for IN-exclusive administration. In addition, long-lasting follow-ups to explore histological variables and the progression of all variables in order to detect late responders are ongoing. Finally, the present work contributes to the clinical experience with the product in a non-Asian population and support the use of HeberNasvac as part of a sanitary intervention, a novel tool to control CHB to accomplish with WHO agenda to control viral hepatitis by 2030.

Authors' Contributions

JCAR, FMFA, VM, GL, and GGN conceived and designed the experiments; FMFA, JAS, GF, EC, and CRI performed the experiments; ZC, NF, and PAD collected the samples; FMFA, JCAR, VM, GL, EPA, GGN, and MC analyzed and interpreted the data; FMFA and JCAR wrote the manuscript.

Ethics Approval

The study received the approval of the Ethical Committee from each of the six Hospitals involved in the trial, and the authorization of the Cuban Center for the State Control of Medicaments (CECMED).

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