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The Effect of Oral Levamisole Co-administration on the Level of Immune Response to Hepatitis B Vaccine in Healthy Individuals: A Randomized Clinical Trial

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ABSTRACT

Despite its proven efficacy, the hepatitis B vaccine requires improvements in immune enhancement and durability, especially in the elderly. Levamisole, an immune modulator, has been tested as an adjuvant to hepatitis B vaccine in several studies in immune-compromised populations. However, we aimed to evaluate the effect of levamisole on the immune response to hepatitis B vaccine in healthy subjects.

In this randomized clinical trial, healthy family members of chronic hepatitis B patients were given twenty-microgram intramuscular injections of hepatitis B vaccine at 0, 1, and 6 months and 50 miligrams of oral levamisole twice a day for two weeks with every vaccination dose. Serum hepatitis B surface antibody (HBsAb) levels of ultimately 98 individuals were measured one month after the final vaccination dose and compared to those of 119 subjects that received placebo and vaccine with an identical regimen. HBsAb levels >10 mIU/mL were considered protective. The Student's t-test, Mann-Whitney test, Kruskal–Wallis analysis (quantitative comparison in age groups), Chi-square test, and the Pearson correlation were used to analyze data. $p<0.05$ was considered significant.

Serum HBsAb levels were significantly higher in the test group ($p<0.001$). All test subjects had levels above 50 mIU/mL (86.7% exceeding 100 mIU/mL). The quantitative response according to age groups was remarkable ($p=0.01$ and $p<0.001$ for placebo and levamisole, respectively), while that of gender was insignificant ($p=0.9$). Unlike HBsAb titers amongst controls, levels in the levamisole group were affected by smoking ($p=0.79$ and $p=0.006$, respectively).

We conclude that oral levamisole as an adjuvant to the hepatitis B vaccine enhances the anti-HBs antibody in healthy vaccinees.

Keywords: Immunization; Hepatitis B vaccines; Levamisole

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INTRODUCTION

The infection caused by hepatitis B virus is a major health issue throughout the world. Hepatitis B vaccination is the major means of controlling the disease and saves both money and lives.[1,2]

The highest incidence of hepatitis B infection in the United States occurs in adults aged 25-45 years.[3] In many developing countries (e.g. Iran), following effective vaccination programs, the prevalence of chronic hepatitis B infection has decreased among children and young adolescents and the adult population has the highest incidence of this infection.[4] More than 90% of those <40 years develop immunity following appropriate vaccination (≥3 doses of hepatitis B vaccine). However, this figure decreases to below 90% for those above 40 years of age and to only 75% in those above 60 years.[4] As a result, an average of 10-25% of adult vaccinees may not develop protective antibody levels. Considering the large number of adults who are vaccinated, this figure amounts to a significant number of individuals who despite a≥3-dose vaccination course may not be immunized. Given that many adults undergo routine age-based rather than risk factor-based vaccination or might even receive vaccination merely upon request, post vaccination serology testing is not routinely recommended for all adults; it is recommended only in those whose subsequent clinical management depends on knowledge of their immune status. Therefore, many unimmunized vaccinees will go undetected, assuming they are immunized, especially when a single 3-dose vaccination protocol is the norm. This may be regarded as significant considering the major complications of infection with the hepatitis B virus, most notably cirrhosis and hepatocellular carcinoma.[5]

Nevertheless, it may be possible to perform routine post vaccination testing in all adults who undergo vaccination with 3 doses of the hepatitis B vaccine. However, this approach is flawed by the fact that hepatitis antibody testing in all vaccinees may not be cost-effective due to the high costs of both the serologic test and the second series of vaccination once tests reveal insufficient response to the initial series of vaccination. Besides, an enhanced immunogenicity of the first initial doses of the vaccine will potentially obviate the need for further testing and repeat vaccine doses and eliminate the possible inefficiencies observed in many clinical settings regarding further testing and vaccination. Therefore, enhancing the immunity-inducing capability of the initial three doses of the vaccine seems to be a rational approach to decreasing or even eliminating the proportion of vaccines for those, who do not develop immunization following 3 doses of hepatitis B vaccine. Currently, guidelines recommend HBs Ab testing in high risk populations and a repeat 3-dose vaccine series in those who do not develop immunization following the initial 3 doses.[6]

In recent years there has been a trend towards promoting the immune response to the hepatitis B vaccine in the elderly and immune compromised via the use of immune-enhancing adjuvants, amongst which levamisole has been of particular interest due to low costs and a relatively safe side-effect profile. This antihelminthic drug is an immune modulator, which belongs to the synthetic imidazothiazole derivatives. It is believed to increase and activate T-cells through the enhanced production of IL-1, IL-2, IL-12,and IL-18.[7] In humans, it has also been used in the treatment of certain types of cancer, as combination therapy for influenza infection, for a range of dermatologic conditions, and many other diseases .[8] The drug has been tested as an immune enhancer in hemodialysis and human immunodeficiency virus (HIV) patients undergoing hepatitis B vaccination in several clinical trials with conflicting results.[9-14] Since screening for nonresponders is not cost-effective in all cases of vaccination and the durability of the vaccine-induced protection is undefined in normal individuals, the purpose of the present study was to evaluate the effect of levamisole as an adjuvant to the hepatitis B vaccine on the immune response in healthy individuals.

MATERIALS AND METHODS

Study Design

This study was a randomized clinical trial. The study population consisted of 217 healthy family members of hepatitis B patients referring to the hepatology clinic at Ghaem Hospital, Mashad, Iran from May 2014 to June 2014. Since hepatitis B vaccinations are generally conducted in high risk adult populations, and many adults in Iran have not been vaccinated, healthy family members of hepatitis B patients were chosen because they represent such populations. All participants had been provided with written informed consent sheets stressing the possible dose-dependent side effects of levamisole therapy. The

study was approved by the Ethical Committee of Mashad University of Medical Sciences (No. IR.MUMS.REC.1393.76).

All those who had consented to the study underwent testing for Hepatitis B surface antigen (HBsAg) and HBsAb levels. In addition, a thorough history was obtained to confirm a negative personal history of hepatitis B vaccination. Only those with a negative history of hepatitis B vaccination, negative HBsAg, undetectable HBsAb levels, and negative hepatitis B core antibody (HBcAb) tests were included. The exclusion criteria consisted of age above 60 years, a history of current or past malignancy, immunosuppressive medication use, detectable HBsAb levels and/or positive results for HBc Ab, and/or liver disease, including positivity for hepatitis C virus antibody (HCVAb). Furthermore, patients would be excluded if they developed side effects related to levamisole therapy during the study period.

Out of the 364 family members of hepatitis B patients referring to the hepatology clinic over a 2-month period, 244 healthy subjects met the criteria for enrollment into the study. 120 individuals were excluded because of various reasons including; a positive history of hepatitis B vaccination (32), age (29), history of malignancy (4), liver disease (3), HBs Ag positivity (15), HBc Ab positivity (1), HBs Ab positivity (34) or merely a lack of consent (2). However, the total number of participants who completed the study was 217 (137 subjects were partners, 42 subjects were offsprings, and 38 subjects were siblings). This study was registered in the Iranian Registry of Clinical Trials (IRCT registration number: IRCT201 4110618915N2)

Intervention

A table of random numbers was applied to allocate the subjects enrolled in the study into a test and a control group. The people in charge of enrolling the participants and those assigning every subject into either group were blinded to the study. Each participant in either of the two groups received three 20-microgram injections of the hepatitis B vaccine (Euvax B, LG Chemicals, Korea) into the deltoid muscle at 0, 1, and 6 months. In addition to the three hepatitis B vaccine doses, the individuals in the test group also received 50 mg of oral levamisole (50 mg tablets, Ruzdaru, Iran) every 12 hours for 14 days starting one week prior to the administration of every vaccine dose (i.e., three two-week courses of levamisole therapy). Subjects in the control group were given placebo with similar dosing intervals. All placebo tablets were manufactured with an identical shape and size and placed in containers identical to those of levamisole tablets.

An adequate heparinized blood sample (5 cc) was drawn by venipuncture from every subject and manually labelled. Plasma or serum was prepared using standard techniques. Visibly hemolysed (red) or hyperlipimc (milky) samples were discarded. Samples were stored at +2-8^{0}C and analyzed within 5 days after collection. In the collected samples there were no samples with antibody titers above the maximum detectable range of the ELISA kit used.

Hepatitis B antibody titers, HBs Ag and HBc Ab levels were measured using the ELISA (Dia.Pro. Diagnostic Bioprobes Srl. via G.carducci n^{o}27-Sesto Scan Giovanni (MI), Italy) at the reference laboratory of Ghaem Hospital one month after the administration of the third dose of the vaccine.

Given the rare but serious side effect of levamisole i.e. agranulocytosis, which has been mostly reported following abuse of levamisole-adulterated cocaine,[15,16] all subjects were instructed to present to the clinic if they encountered any flu-like symptoms (e.g., fatigue, fever, rigors, malaise, headache, cough) and were examined for such side effects during visits for the second and third vaccination doses and at the time of the final presentation for testing of serum HBsAb levels. Subjects were also required to inform the clinic by phone upon any suspicious symptoms regarding other potential levamisole side effects (e.g., rash, nausea, diarrhea, vomiting, depressed mood, blurred vision). For allocation concealment, subjects in charge of vaccinations and follow-ups for possible drug side effects were blinded to the study. Also enquired during visits was the correct use of oral therapy.

Those vaccinees with an antibody level of>10 mIU/mL were considered responsive. However, the proportion of vaccinated individuals with levels ≤10 mIU/mL became candidates for a second 3-dose vaccination series based on international protocols for non-responders.[6] In the present study, an antibody titer of <10 mIU/mL was considered as not protective.

Principle of the HBs Ab Test

Microplates coated with highly purified HBsAg capture sample anti HBsAg antibodies. After washing,

captured antibodies are detected by an HBsAg labeled with peroxidase (Sigma-Aldrich, Germany). The specifically bound enzyme generates an optical signal that is proportional to the amount of HBsAb in the sample and be detected by an ELISA reader. Quantification of antibodies is performed by means of a standard curve calibrate against the WHO reference preparation.

Statistical Analysis

Results were expressed as mean±standard deviation for continuous variables. Comparisons between the groups were made using the Student's t-test for continuous variables and X2 test or Fisher's exact test, when appropriate, for categorical variables. The Kruskal–Wallis test was applied for quantitative comparison in age groups. The Chi-square test and the Pearson correlation were applied to compare the level of anti-HBs antibody in the two groups. The normal distribution was checked by the Kolmogorov–Smirnov test.

Results with a p value of<0.05 were considered significant. Data analysis was done using the Statistical Package for the Social Sciences (SPSS version 11.5, SPSS Inc, Chicago, Ill, USA).

RESULTS

The total number of participants who completed the study was 217, of which 102 were female and 115 were male. At the end of the study, out of the initial number of subjects, who had been randomly divided into a levamisole and a placebo group, 98 and 119 vaccinees remained in each group, respectively. The mean age in the placebo and levamisole groups was 32.54±1.14 years and 33.77±1.27 years, respectively (p=0.642). The relevant demographic data are presented in Table 1.

The cause for the failure to complete the study for 25 individuals (10 male and 15 female) was merely a lack of presentation for the follow-up doses of the hepatitis B vaccination and/or levamisole administration or incorrect oral therapy dosing, despite persistent reminders via phone and/or e-mail. Two other male subjects from the levamisole group (aged 35 and 42 years) were excluded from the study following the development of nausea and vomiting attributed to oral levamisole intake.

All subjects in the levamisole group responded to the vaccination with antibody levels above 50 mIU/mL. However, out of 119 participants in the placebo group, 22(18.5%) did not acquire protective antibody levels (i.e., >10 mIU/mL) after 3 doses of hepatitis B vaccine. An additional 8 (6.7%) vaccinees, despite achieving levels considered to be protective, did not develop antibody titers in excess of 50 mIU/mL. Interestingly; however, antibody levels above 100 mIU/mL were observed in the majority of the participants in the levamisole group (90.8%), of whom over 10% achieved levels beyond 200 mIU/mL. On the contrary, levels exceeding 100 mIU/mL were detected in only 37.8% of the volunteers in the placebo group, of whom less than 5% had measurements above 200 mIU/mL.

Overall, a significant difference was observed in the level of response to the hepatitis B vaccine between those treated with levamisole and those receiving placebo (p<0.001). The levels of anti-HBs antibody in both the levamisole and control groups are presented in Table 2.

Table 1. Age and sex characteristics along with the status of cigarette smoking and opium abuse in both the subjects receiving levamisole and hepatitis B vaccine and those receiving placebo with the vaccine

Category		Placebo n*(%)	Levamisole n(%)	*p*-value
Sex	Female	56(47.05)	46(46.94)	0.99
	Male	63(52.94)	52(53.06)	
Age (years)	<20	12(10)	8(8.2)	0.64
	20-39	69(58)	53(54.1)	
	≥40	38(32)	37(37.8)	
Smoking status	Smoker	43(36.1)	34(34.7)	0.82
	Non-smoker	76(63.9)	64(65.3)	
Addiction to opium	Yes	9(7.6)	6(6.1)	0.67
	No	110(92.4)	92(93.9)	

*n: number

Table 2. The level of anti-HBs antibody in response to the hepatitis B vaccine in the group receiving levamisole and the group given placebo

Category \ Serum Antibody Level (mIU/mL)	≤10/non-protective n*(%)	11-49 n(%)	50-99 n(%)	100-199 n(%)	≥200 n(%)	*p*-value
Placebo	22(18.5)	8(6.7)	44(37)	43(36.1)	2(1.7)	<0.001
Levamisole	0(0)	0(0)	9(9.2)	79(80.6)	10(10.2)	

*n: number

Although antibody levels were higher amongst females, the assumptions for the Chi-square test, based on the antibody-level categories provided in Table 3, were not met for gender or age. However, when antibody titers were analyzed quantitatively in both the placebo and levamisole groups a significant difference was observed regarding the effect of age (placebo group and levamizole group; p=0.01 and p<0.001, respectively). In addition, the subjects' age was found to be negatively correlated with antibody levels in both the levamisole (Pearson Correlation=-0.389; p=<0.001) and control (Pearson Correlation=-0.227; p=0.013) groups. Unlike 'age', 'gender' did not significantly alter the immune status of hepatitis B vaccinees in either the placebo or levamisole group (p=0.3 and p=0.9, respectively).

Furthermore, no influence of gender or age was seen when the population of individuals responding (i.e., HBsAb >10 mIU/mL) to the vaccine and placebo were compared to their non-responding (i.e., HBsAb ≤10 mIU/mL) counterparts (Table 4).

Cigarette smoking did not have an effect on the anti-HBs antibody levels to the hepatitis B vaccine plus placebo (p=0.79, Student's t-test; 68.57±44.56 mIU/mL and 84.61±48.87 mIU/mL for smokers and nonsmokers, respectively). This effect; however, was remarkable when levamisole was co-administered with the vaccine (p=0.006, Student's t-test; 129±27.30 mIU/mL and 149.50±36.27 mIU/mL for smokers and nonsmokers, respectively). With regard to addiction, there were not enough cases to perform a statistically valid analysis.

Table 3. The frequency distribution of the level of anti-HBs antibody according to gender and age in the group receiving levamisole and hepatitis B vaccine and the control group receiving placebo and the vaccine

Category \ Serum Antibody Level (mIU/mL)			≤10 n*(%)	11-49 n(%)	50-99 n(%)	100-199 n(%)	≥200 n(%)
Placebo	Sex	Female	12(54.5)	4(50)	15(34.1)	25(58.1)	0(0)
		Male	10(45.5)	4(50)	29(65.9)	18(41.9)	2(100)
		Total	22(100)	8(100)	44(100)	43(100)	2(100)
	Age	<20	2(9.1)	0(0)	2(4.5)	8(18.6)	0(0)
		20-39	16(72.7)	2(25)	22(50)	27(62.8)	2(100)
		>39	4(18.2)	6(75)	20(45.5)	8(18.6)	0(0)
		Total	22(100)	8(100)	44(100)	43(100)	2(100)
Levamizole	Sex	Female	0(0)	0(0)	3(33.3)	37(46.8)	6(60)
		Male	0(0)	0(0)	6(66.7)	42(53.2)	4(40)
		Total	0(0)	0(0)	9(100)	79(100)	10(100)
	Age	<20	0(0)	0(0)	0(0)	5(6.3)	3(30)
		20-39	0(0)	0(0)	3(33.3)	43(54.4)	7(70)
		>39	0(0)	0(0)	6(66.7)	31(39.2)	0(0)
		Total	0(0)	0(0)	9(100)	79(100)	10(100)

*n: number

Table 4. A comparison between individuals with and without a sufficient anti-HBs antibody response to a combination of 3 doses of hepatitis B vaccine and placebo based on gender and age

Category	Serum Antibody Level (mIU/mL)	≤10 mIU/mL n*(%)	>10 mIU/mL n(%)	*p*-value
Sex	Female	12(54.5)	44(45.4)	0.436
	Male	10(45.5)	53(54.6)	
Age	<20	2(9.1)	10(10.3)	0.268
	20-39	16(72.7)	53(54.6)	
	>40	4(18.2)	34(35.1)	

*n: number

Levamisole therapy was well tolerated by almost all the participants with no drug-related side effects. There were two vaccinees that developed gastrointestinal symptoms of nausea and vomiting following administration of levamisole.

DISCUSSION

The most significant finding of the present study was the higher level of HBsAb in the sera of those healthy vaccinees that had received levamisole in addition to the hepatitis B vaccine. Also worth noting was the fact that not only did all vaccinees in this group acquire protective antibody titers (i.e., HBsAb>10mIU/mL), but also all subjects were found to have HBsAb levels above 50 mIU/mL, with an overwhelming majority exceeding 100 mIU/mL one month after the 3rd vaccine dose.

In a study by Sayad et al, it was shown that levamisole co-administration in HIV patients receiving the hepatitis B vaccine was beneficial in increasing the immune response to the vaccine.[9] In a similar clinical trial performed on hemodialysis patients; however, Sanadgol et al did not confirm any difference between those receiving levamisole as an adjuvant to the hepatitis B vaccine and those receiving the hepatitis B vaccine plus placebo.[10] It was noted by the authors that the type of vaccine together with the timing of administration and dosage of levamisole may have influenced the results, which were contradictory to other studies. Similar results were also obtained by another study performed by Sali et al, in that no beneficial effect was reported on the immune enhancement caused by levamisole treatment in hemodialysis patients, who had an acceptable response to hepatitis B vaccination alone.[11] However, it should be emphasized that the response rate to the routine vaccination (i.e. without levamisole administration) amongst the hemodialysis patients in the latter study was above 80%, a figure at least 20% higher than the average response rates reported by other studies. In contrast to the results in the two studies mentioned and in line with most reports on the immune enhancing effect of levamisole, a meta-analysis by Alavian and Tabatabai, including 4 studies and 328 patients, revealed a significant benefit of the administration of levamisole in stimulating the immune response in end-stage renal disease patients.[12] Arghani and Shojai had also concluded that levamisole administered together with intradermal injections of the hepatitis B vaccine could be the best approach to immunizing patients on chronic hemodialysis and that it may increase the durability of the vaccine-induced immunization.[13] Previously in 2002, the beneficial role of levamisole treatment in hemodialysis patients had also been demonstrated by Kayatas.[14]

In all the mentioned studies and others, the effect of levamisole was evaluated in immunocompromised patients. However, there has not been any research in the literature, evaluating the effect of levamisole treatment on the immune response to the hepatitis B vaccine in healthy individuals. It has been estimated that 5-15% of the normal adult population, uninfected by the hepatitis B virus, who undergo hepatitis B vaccination do not respond to the vaccine after full 3-dose series and are therefore called non-responders.[17]

The percentage of vaccinees without a sufficient response (i.e., ≤10 mIU/mL) to the initial 3 vaccine doses in our study was 18.5%. It demonstrated a higher non-responder rate (following three vaccine doses) than

most other studies. According to a study by Kardar et al, T-helper 1 and T-helper 2 imbalance due to lack of cytokine (interleukine [IL] 2, IL10, and interferon gamma) production was suggested as a cause for lack of antibody production.[18] In another study, inadequate production of HBsAg-specific precursor B cells was reported as a cause.[19] Janbakhsh et al reported a 69.6% response rate in health care workers in Kermanshah, a figure significantly lower than those presented in the literature. The researchers reported low vaccine quality as the major contributing factor.[20] In a similar study by Zamani et al, HBsAb titers <10 mIU/ml following vaccination were reported in 17.2% of vaccinees. However, no significant relationship was reported between response rates and age/gender.[21] In our study, the low response rate to vaccination in the placebo group may be due to low level viremia following long-term exposure especially amongst partners.[18] In addition, subclinical infections in developing countries such as Iran may contribute to an altered immune response.[22] Other factors such as genetic predisposition (the haplotype of HLA antigens), race, vaccine type, and technique may play a role in lower response rates.[23-25]

However, the fact that all the participants in the levamisole group had responded efficiently to the vaccination and that antibody levels most frequently found in this population were > 100 mIU/mL may be indicative of the efficiency of levamisole co-administration when it comes to inducing a sufficient immune response.

In an article by Platkov et al in 2001, an association was demonstrated to exist between the time lapse after vaccination and the level of antibody response.[29] Of the total number of healthy hospital employees vaccinated against HBV, 13.5% had insufficient antibody levels (i.e. ≤10 mIU/mL) detected after 5 years post-vaccination. The authors had expressed concern about the high prevalence of unprotected individuals who had received the complete standard vaccination against HBV. Other studies had also suggested that HBs Ab titers decrease over time.[27]

These reflected the fact that antibody levels < 100 mIU/mL were associated with an earlier loss of protective antibody levels in the sera of such patients and as a result suggested the need to maintain levels above 100 mIU/mL.[28] In healthy individuals; however, there is more of a controversy over the waning of protective antibody levels over time. Studies performed on healthy adolescents with conventional vaccination protocols since infancy have revealed contradictory results as to the immune status of this age group. A study in Taiwan revealed that 29% of the students with undetectable antibody levels after 15 years still lacked sufficient response following a single booster dose.[29] A study from Alaska reported that almost half (49%) of the 15-year-olds that had received vaccinations during infancy showed no response after a booster dose.[30] Both of the studies and others were suggestive of a loss of an immunological memory even in young and healthy individuals.

Therefore, it may be rational to think that the higher the primary immune response to the vaccine, the more durable the immunized state. This may be a case in favor of levamisole treatment during hepatitis B vaccination since we showed that individuals receiving levamisole treatment have higher post-vaccination antibody titers.

Another factor predicting both the response rate and durability of protective antibody titers is age. According to a study by Looney et al elderly vaccinees demonstrated a significantly lower rate of protective immune response than their younger counterparts.[31] Overall, most guidelines and the results in our study both in the control and test groups suggest an inverse relationship between age and the immune response to hepatitis B vaccine. This; however, was not found to be the case when Ibrahim S et al evaluated antibody titers post-vaccination in hemodialysis patients.[32]

Regarding the effect of gender on antibody levels, no significant effect was observed in our study. In a study on 144 hemodialysis patients, Al Saran et al demonstrated similar unremarkable results.[33] These results and others, including those obtained in the present study, were in contrast to the Centers for Disease Control and Prevention information, where the male gender was reported as a risk factor for lack of a sufficient immune response.[34]

Finally, smoking has been reported as a risk factor in curtailing an immune response following hepatitis B vaccination.[26,35,36] In our study, this effect was significant in the levamisole group, whereas no statistical significance was shown in the population receiving the vaccination and placebo.

The limitations of the study included a lack of objective proof as to the compliance of patients in taking levamisole and the fact that the results were not applicable to those over 60 years of age. Also, the

available data regarding the effect of addiction were insufficient to make any conclusions. In addition, checking antibody levels prior to the administration of the third dose of the vaccine (i.e. following the first or second doses) in those patients receiving levamisole therapy may provide data regarding acquiring protective levels with only one or two doses of vaccine. Furthermore, since levamisole effect on the anti-HBs antibody levels to the hepatitis B vaccine had not been evaluated in healthy individuals prior to the present study; comparisons could not be made reliably. It is worthwhile to consider HBV DNA testing in non-responders who may have occult infection not detected through routine serologic tests. However, such detection approaches are not considered regularly.

The results from the present study are suggestive of an immune-enhancing role for the drug, levamisole, in healthy individuals vaccinated for hepatitis B. Further studies are required in this regard.

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