

Therapeutic and immune function improvement of vitamin D combined with IFN- α on mouse with hepatitis B infection

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Abstract

This study aims to investigate the therapeutic effect of vitamin D (VD) combined with interferon (IFN) type I (IFN- α) on mice with hepatitis B and to explore the possible mechanism. The mice were divided into control group, model group, IFN- α group, and IFN- α +VD group. After 4 weeks, the mice of four groups were weighed, and the thymus and spleen indexes were calculated. The activity of alanine transaminase (ALT) and aspartate transaminase (AST) in the serum was detected while pathological changes of the liver were observed. The levels of interferon- γ (IFN- γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) in serum were measured by enzyme-linked immunosorbent assay (ELISA). The proliferation of spleen lymphocytes was detected by MTT assay. Flow cytometry was used to detect the level of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ in peripheral blood. The levels of ALT and AST in serum were significantly lower in the IFN- α +VD group than those in the IFN- α group, but the thymus and spleen indexes were significantly higher. Although both IFN- α and IFN- α +VD can improve the damaged structure of live tissue, IFN- α +VD achieved higher efficacy than IFN- α alone. The serum IFN- γ , TNF- α , and IL-2 levels were lower in the IFN- α +VD group compared with the IFN- α group, and no significant difference was found in IL-4. Compared with the IFN- α group, the percentage of CD4⁺ and the D4⁺/CD8⁺ ratio were significantly increased, but the percentage of CD8⁺ was reduced. The proliferation rate of splenic lymphocytes was higher in the IFN- α +VD group compared with the IFN- α group. IFN- α +VD was found to achieve higher efficacy than IFN- α alone for the treatment of hepatitis B in mice, possibly through increasing the immune level of mice.

Keywords

25-hydroxy-vitamin D, CD4⁺, CD8⁺, hepatitis B, IFN- α

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Introduction

Hepatitis B is a crucial public health issue all over the world nowadays, which is caused by hepatitis B virus (HBV) infection.¹ In China, although the administration of HBV vaccination has dramatically reduced the prevalence of hepatitis B, the incidence rate is still as high as 7%–8% in rural China.² The functional impairment of HBV-specific T-cells is the main pathological feature of HBV infection.³ Based on the therapy of restoring the ability of HBV-specific T-cell, interferon- α (IFN- α); has been widely used in the treatment of hepatitis B.³ It is well accepted that IFN can participate in the viral gene expression to perform the functions of anti-virus

and immune regulation.⁴ However, this therapy is still challenged by the side effects, including flu-like syndrome, fatigue, bone marrow suppression,

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depression-like psychiatric symptoms, and so on.⁵ Therefore, highly effective drugs and treatments to alleviate these side effects have become critical to minimize the popularization of IFN.

Vitamin D (VD) is a group of secosteroids which can participate in a variety of signaling pathways to regulate the body's immune system and act in a similar way to cytokines.⁶ VD is an important regulator of the migration and homing of T-lymphocyte and also plays pivotal roles in the T-lymphocyte differentiation and induction of immune tolerance.⁷ The regulation of VD for the differentiation of T-lymphocyte can benefit the maintenance of the dynamic balance between CD4⁺ and CD8⁺, which in turn improves the normal immune response, so as to maintain the relative balance of the immune response and the body's normal immune status. Recent studies have shown that the low level of VD in serum of patients with hepatitis B is one of the main causes of the high replication level of hepatitis B.⁸ So VD treatment is believed to be beneficial for the recovery of hepatitis B and it will be reasonable to hypothesize that the application of VD substitutes for IFN treatment of hepatitis B to improve the treatment outcomes.

In this study, VD combined with IFN type I (IFN- α) was used to treat the mice hepatitis B model with IFN- α as a control. The treatment efficacies of two methods were compared at multiple levels.

Materials and methods

Experimental animals

Specific-pathogen-free (SPF) grade HBV transgenic and non-transgenic BALB/c mice (6–8 weeks old, body weight range from 18 to 22 g, half male and half female) were purchased from the 458th Hospital of People's Liberation Army. All animal experiments have been approved by the Animal Ethics Committee of our institute.

Animal grouping and treatment

The mice were divided into four groups (n=10 per group). The mice in control group were non-transgenic BALB/c mice, and all the mice in this group were subjected to intragastric administration of 0.9% saline, 0.2 mL for each day. The mice in model group were all HBV transgenic BALB/c mice and

were subjected to intragastric administration of 0.9% saline, 0.2 mL for each day. The mice in the IFN- α group were also HBV transgenic BALB/c mice, and they were subjected to intramuscular injection of IFN- α (5 million unit per time per day). All HBV transgenic BALB/c mice in the IFN- α +VD group were subjected to intramuscular injection of IFN- α (5 million unit per time per day) and intragastric administration of VD (0.03 μ g/kg/day). The above administration was continued for 4 weeks.

Specimen collection and treatment

After 4 weeks of administration, the mice were sacrificed and the body was weighed. Blood was taken from the eyes. The spleen and thymus were taken and weighed, and thymus index and spleen index were calculated. And then the liver tissue was placed in 4% neutral formaldehyde solution for pathological analysis, and spleen was used to prepare cell suspension.

Biochemical indicator detection

The blood samples were collected from the mice in each group. The activity of aspartate transaminase (AST) and alanine transaminase (ALT) in serum of each group was detected by XL-600 automatic biochemical analyzer (Transasia, Mumbai, India).

Histopathological examination

The liver tissue was fixed in 4% neutral formaldehyde solution for 24 h, and then dehydration was performed by passing the tissue at a series of increasing alcohol concentrations. After paraffin embedding, the tissue was cut into slices with a thickness of 5 μ m. The tissue was then transferred onto the glass slides and hematoxylin-eosin (HE) staining was performed using a kit (Beyotime Institute of Biotechnology, Jiangsu, China). The histopathological changes were observed under an optical microscopy (Olympus BX51; Olympus, Tokyo, Japan).

Determination of thymus and spleen indexes

Thymus and spleen were taken and weighed after mice were sacrificed, and thymus index and spleen index were calculated. Computation formula is as follows

$$\text{Thymus index (mg/g)} = \frac{\text{weight of mice thymus (mg)}}{\text{body weight (g)}}$$

$$\text{The spleen index (mg/g)} = \frac{\text{weight of mice spleen (mg)}}{\text{body weight (g)}}$$

ELISA to detect the levels of IFN- α , tumor necrosis factor α , interleukin 2, and interleukin 4 in serum

After 4 weeks of administration, the eyeballs of the mice were removed and the blood was collected. The levels of cytokines IFN- γ , tumor necrosis factor α (TNF- α), interleukin 2 (IL-2), and interleukin 4 (IL-4) in serum were measured by ELISA according to the instructions of ELISA kit (R & D Co. Ltd., Minneapolis, MN, USA).

Flow cytometry to detect the T-lymphocyte subgroups in peripheral blood of mice

The above collected blood was added into three test tubes with 100 μ L in each. After that, 3 μ L of anti-mouse CD4-FITC/CD8-PE/CD3-PerCP conjugate antibody (BD, New Jersey, USA) and isotype control antibody were added, respectively. Then, the mixture was incubated at room temperature in dark for 15–20 min. And then 2 mL 1 \times lysing solution (Becton Dickinson, Heidelberg, Germany) was added into each tube and the mixture solution was incubated at room temperature in dark for 5–10 min, followed by centrifugation. The cells were first suspended and washed with 1 \times phosphate-buffered saline (PBS, containing 0.5% bovine serum albumin (BSA) and 0.1% NaN₃; Beyotime Institute of Biotechnology), followed by centrifugation for 5 min. Then, the obtained cells were resuspended with 1 \times PBS to make a final value of 0.5 mL and BD FACSCalibur flow cytometry detection was performed (Becton Dickinson). The percentage of CD4⁺ and CD8⁺ cells in T-cells and the CD4⁺/CD8⁺ ratio were calculated using FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of splenic lymphocyte proliferation by MTT assay

Spleen was taken under sterile conditions and was grinded on a copper mesh (200 mesh) at 4°C. After filtration, Hank's solution (Invitrogen-Gibco,

Carlsbad, CA, USA) was added and the mixture was transferred into the sterilized centrifuge tube, followed by centrifugation (1500 r/min) for 10 min. The supernatant was removed, and the centrifugation was repeated three times. The cell culture medium was added to make cell suspension. The cell suspension was then counted using a blood cell count plate and the cell density was adjusted to 5 \times 10⁶ cells/mL.

The cells were then inoculated into a 96-well cell culture plate, 0.8 \times 10³ cells/well, and concanavalin A (Con A; Sigma-Aldrich, St Louis, MO, USA) was added, which made a final concentration of 5 μ g/mL. RPMI1640 medium containing 100 mL/L calf serum was used as the blank control. Three replicate wells were set for each sample. The cells were cultured in an incubator containing 5% CO₂ for 72 h. MTT solution (50 μ L, 2 mg/mL) was added at 4 h before the end of the incubation. After incubation, the supernatant was removed and 150 μ L of dimethyl sulfoxide (DMSO) was added into each well and gently shook for 15 min. The optical density (OD) value was measured using a microplate reader (Perkin Elmer®, Waltham, MA, USA) at 570 nm, with the OD value of the control well set as 0.

Statistical analysis

SPSS 19.0 statistical software was used to analyze the variance of each monitoring data. All the data were expressed as mean \pm standard deviation (\pm SD). The t-test was used for the comparisons between two groups. One-way analysis of variance (ANOVA) was used for the comparisons among more than two groups, and $P < 0.05$ was considered to be statistically significant.

Results

Effects of different treatments on serum ALT and AST in mice

The levels of serum ALT and AST in each group were detected. The results showed that, compared with the control group, the levels of serum ALT and AST in the model and IFN- α group were significantly increased ($P < 0.05$). However, no significant differences were found between the control group and the IFN- α +VD group. Compared to the model group, the levels of serum ALT and AST in the IFN- α group as well as the IFN- α +VD group were significantly

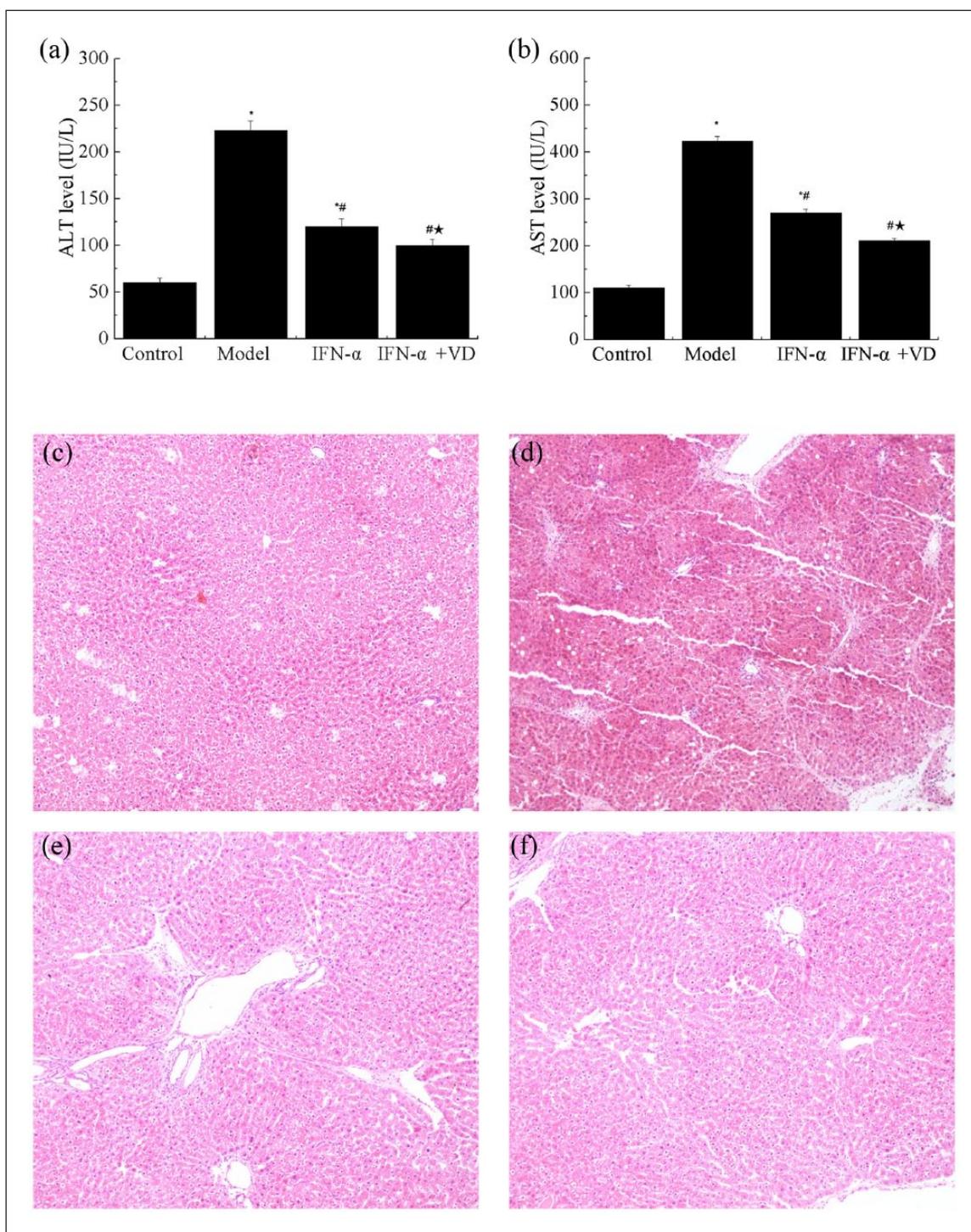


Figure 1. The levels of serum ALT, AST, and HE staining of the liver tissue ($\times 100$) in mice of each group: (a) the levels of serum ALT in mice of each group, (b) the levels of serum AST in mice of each group, (c) liver tissue of the control group, (d) liver tissue of the model group, (e) liver tissue of the IFN- α group, and (f) liver tissue of the IFN- α + VD group. * $P < 0.05$ vs control group; # $P < 0.05$ vs model group; * $P < 0.05$ vs IFN- α group.

decreased ($P < 0.05$). Compared with the IFN- α group, the levels of serum ALT and AST in the IFN- α + VD group were significantly decreased ($P < 0.05$). The results indicated that, compared with IFN- α

alone, IFN- α combined VD treatment had higher efficiency in reducing the increased levels of serum ALT and AST in mice caused by hepatitis B (see Figure 1(a) and (b) for details).

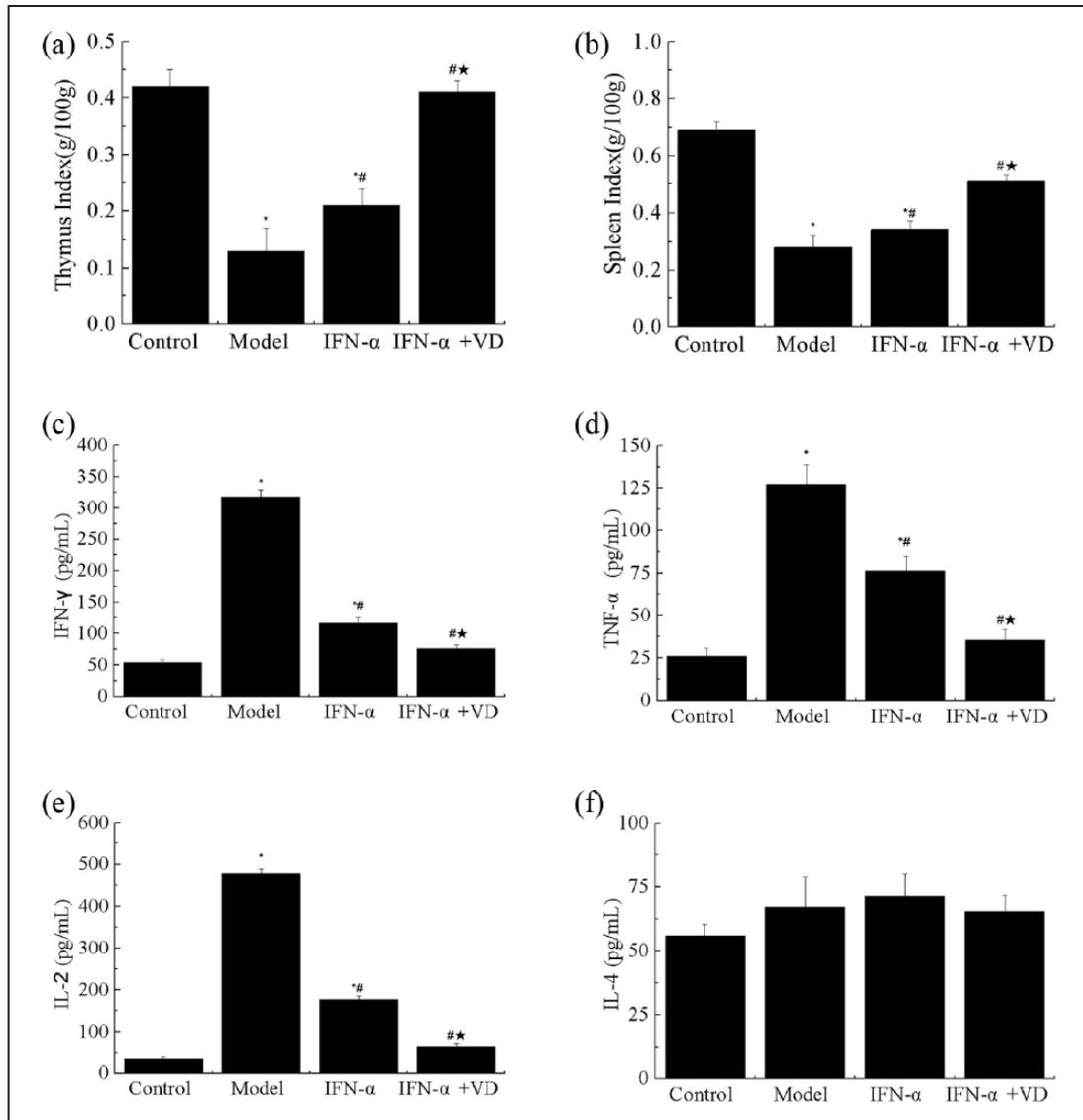


Figure 2. Thymus and spleen indexes, and the levels of IFN- γ , TNF- α , IL-2, and IL-4 in mice of each group: (a) thymus index, (b) spleen index, (c) the level of IFN- γ , (d) the level of TNF- α , (e) the level of IL-2, and (f) the level of IL-4. * $P < 0.05$ vs control group; # $P < 0.05$ vs model group; * $P < 0.05$ vs IFN- α group.

Effects of different treatments on histopathological structures of liver tissues of mice

In the control group, the liver lobules were normal and clear, the liver cells were neat and uniform in size, and the nucleus was located in the center of the cell with round shape and clear boundary, but in the model group, the liver tissue and the structure of the normal hepatic lobule were damaged, the structure of the hepatic sinusoid site was disordered, and diffuse lymphocyte infiltration was also observed between cells. In addition, the neutrophil infiltration occurred in some tissues. Liver cells in

inflammatory sites were balloon-like, and some liver cells showed serious necrosis. The structure of most of the hepatocytes in mice of the IFN- α group and the IFN- α +VD group were intact and neatly arranged. Inflammatory cell infiltration, balloon-like changes of hepatocytes, and the number of death foci were significantly reduced. The liver lesions of the IFN- α +VD group were significantly improved than those in the IFN- α group. Those data suggest that the application of IFN- α +VD can significantly improve the histopathological changes of liver tissues caused by hepatitis B and is more efficient than the application of IFN- α alone (see Figure 1(c)–(f)).

Effects of different treatments on thymus and spleen indexes in mice

The results of thymus and spleen indexes showed that, compared with the control group, the thymus and spleen of the model group and the IFN- α group were significantly lower ($P < 0.05$), and no differences were found between the IFN- α +VD group and the control group ($P > 0.05$). Besides, compared with the model group, the thymus and spleen indexes of the IFN- α group and the IFN- α +VD group were significantly higher ($P < 0.05$). The thymus and spleen indexes were significantly higher in the IFN- α +VD group than in the IFN- α group ($P < 0.05$; Figure 2(a) and (b)). The results suggest that IFN- α +VD can significantly improve the thymus and spleen indexes in mice with hepatitis B, and its efficacy is higher than IFN- α alone.

Effects of different treatments on the expression of IFN- γ , TNF- α , IL-2, and IL-4

The levels of IFN- γ , TNF- α , IL-2, and IL-4 were detected by ELISA. The levels of IFN- γ , TNF- α , and IL-2 in the model group and the IFN- α group were significantly higher than those in the control group ($P < 0.05$), but no significant differences were found between the IFN- α +VD group and the control group. Compared with the model group, the levels of IFN- γ , TNF- α , and IL-2 in the IFN- α group and the IFN- α +VD group were significantly lower ($P < 0.05$). Compared with the IFN- α group, the content of IFN- γ , TNF- α , and IL-2 in the IFN- α +VD group was significantly lower ($P < 0.05$). No difference was found in the level of IL-4 between groups. The results also show that IFN- α +VD has higher efficiency in reducing the expression levels of IFN- γ , TNF- α , and IL-2 than IFN- α alone (see Figure 2(c)–(f)).

Effects of different treatments on lymphocytes subgroups CD4⁺ and CD8⁺ and the CD4⁺/CD8⁺ ratio in peripheral blood of mice

The percentage of CD4⁺ in the model group and the IFN- α group was significantly decreased compared with the control group ($P < 0.05$), while no significant difference was found between the IFN- α +VD group and the control group. Compared with the model group, the percentage of CD4⁺ was significantly increased, the percentage of CD8⁺ was significantly decreased, and the CD4⁺/CD8⁺

ratio was significantly increased in the IFN- α group and the IFN- α +VD group ($P < 0.05$). Compared with the IFN- α group, the percentage of CD4⁺ was significantly increased, the percentage of CD8⁺ was significantly decreased, and the CD4⁺/CD8⁺ ratio was significantly increased in the IFN- α +VD group ($P < 0.05$). The above results indicate that IFN- α +VD was more effective in increasing the percentage of CD4⁺ and decreasing the percentage of CD8⁺ than IFN- α alone (see Figure 3(a)–(d)).

Effects of different treatment on proliferation of splenic lymphocytes

The OD value of the model group and the IFN- α group was found significantly reduced compared with the control group ($P < 0.05$), and there was no obvious difference between the IFN- α group and the control group ($P > 0.05$). Compared with the model group, the OD value of the IFN- α group and the IFN- α +VD group was significantly increased ($P < 0.05$). Compared with the IFN- α group, the OD value of the IFN- α +VD group was significantly increased ($P < 0.05$). Based on those data, IFN- α +VD is considered to have stronger ability in increasing the proliferation of splenic lymphocytes than IFN- α alone (see Figure 3(e)).

Discussion

Previous studies had shown that the strong response of T-cell to multiple epitopes mapped to different regions of the genome of HBV was usually found in patients with severe HBV infection.⁹ The response of the patients to antiviral therapy is evaluated from multiple aspects, including biochemical enhancements (such as ALT and AST), serological recovery (seroconversion to anti-HBe or anti-HBs), and histological improvement (reduced inflammation and fibrosis).

The application of IFN- α has been shown to significantly improve the clinical manifestations, but IFN- α therapy alone is not effective for most patients with chronic HBV infection.^{10,11} Previous studies have shown that the deficiency of VD is closely related to the onset and development of human diseases, and the application of VD can improve the clinical response to therapy.⁷ In our study, VD was used in the IFN- α therapy of hepatitis B and the efficacy was compared with the application of IFN- α alone. It was found that IFN- α +VD had higher efficiency in reducing the increased levels

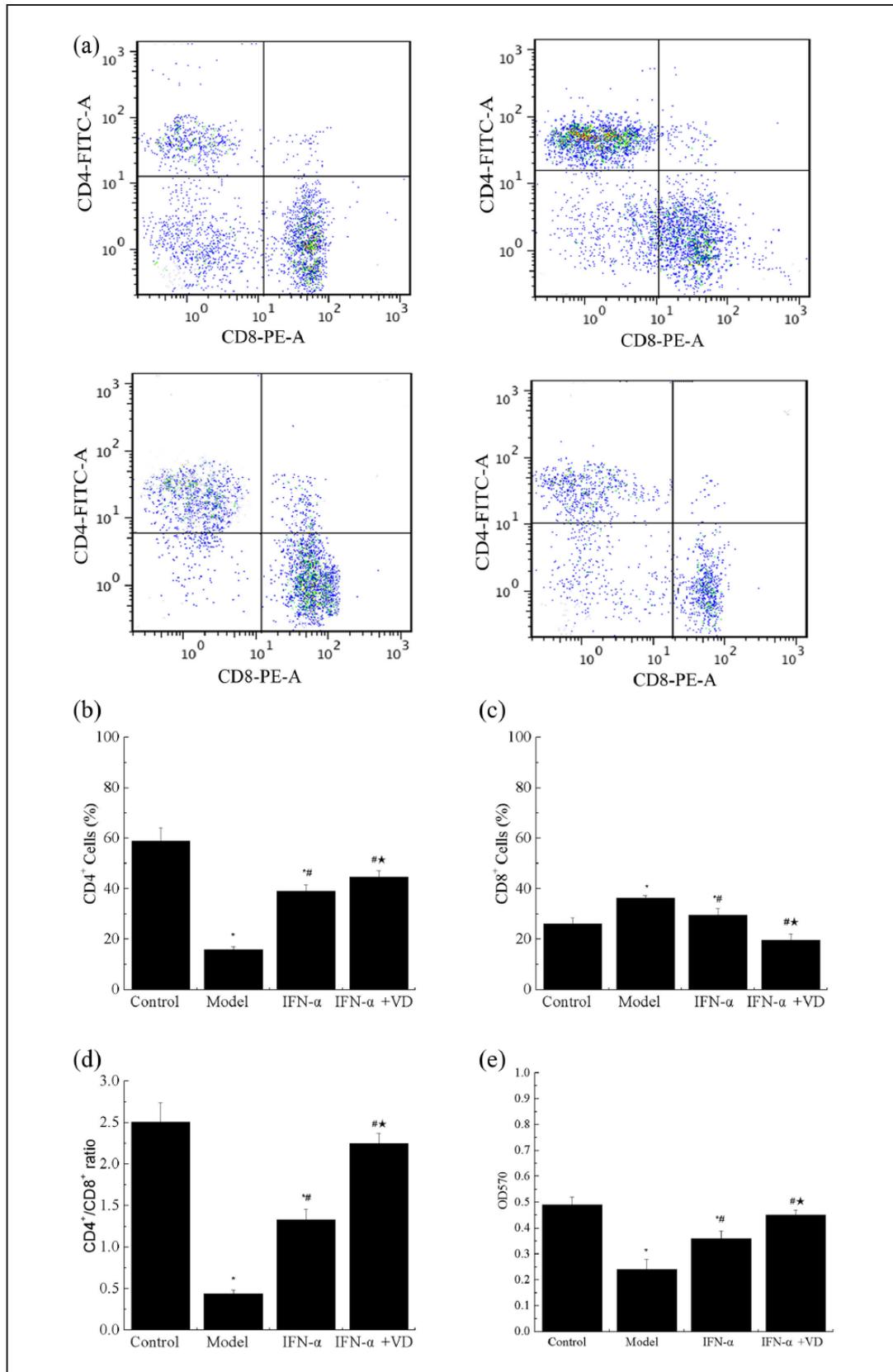


Figure 3. Flow cytometry detection of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ and the proliferation of splenic lymphocytes of each group. (a) Representative flow cytometry results, (b) the percentage of CD4⁺; (c) the percentage of CD8⁺; (d) CD4⁺/CD8⁺ ratio, and (e) the proliferation of splenic lymphocytes. * $P < 0.05$ vs control group; # $P < 0.05$ vs model group; ** $P < 0.05$ vs IFN- α group.

of serum ALT and AST. Also, IFN- α ⁺VD was able to significantly repair the structure of liver tissues caused by hepatitis B and showed a higher efficacy in improving the thymus and spleen indexes, as well as reducing the expression levels of inflammatory cytokines, including IFN- γ , TNF- α , and IL-2. All those data suggest that VD can increase the response of IFN- α therapy to achieve a better therapeutic effect for liver, thymus, and spleen functions and normal liver tissue structures after HBV infection.

The progression of hepatitis B is mediated by the immune system.² This dynamic balance of CD4⁺ and CD8⁺ is important for the maintenance of normal immune responses.¹² In our study, IFN- α ⁺VD was found to be highly efficient in restoring the percentage of CD4⁺ and CD8⁺, and CD4⁺/CD8⁺ ratio to normal levels compared with IFN- α alone. It is well accepted that splenic lymphocytes also play pivotal roles in the maintenance of normal immune functions.¹³ In our study, IFN- α ⁺VD was found to be able to improve the proliferation of splenic lymphocytes. All those data suggest that VD can improve the immune response during IFN- α treatment, which in turn increases treatment efficacy.

In a word, IFN- α combined VD showed a higher efficacy in the treatment of mice with hepatitis B compared with IFN- α alone, and this effect of IFN- α ⁺VD may be achieved through increasing the immune level of mice.

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Y.B. and W.Z. contributed equally to this work.

Declaration of conflicting interests

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