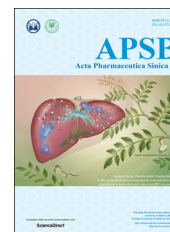




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ORIGINAL ARTICLE

T cell-associated immunoregulation and antiviral effect of oxymatrine in hydrodynamic injection HBV mouse model



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KEY WORDS

HBV;
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Abstract Although oxymatrine (OMT) has been shown to directly inhibit the replication of hepatitis B virus (HBV) *in vitro*, limited research has been done with this drug *in vivo*. In the present study, the antiviral effect of OMT was investigated in an immunocompetent mouse model of chronic HBV infection. The infection was achieved by tail vein injection of a large volume of DNA solution. OMT (2.2, 6.7 and 20 mg/kg) was administered by daily intraperitoneal injection for 6 weeks. The efficacy of OMT was evaluated by the levels of HBV DNA, hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). The immunoregulatory activity of OMT was evaluated by serum ELISA and flow cytometry. Results shows that OMT at 20 mg/kg inhibited HBV replication, and it was more efficient than entecavir (ETV) in the elimination of serum HBsAg and intrahepatic HBcAg. In

Abbreviations: OMT, oxymatrine; HBV, hepatitis B virus; ETV, entecavir; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; HE, hematoxylin and eosin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL-4, interleukin-4; TCMS, traditional Chinese medicines; NAs, nucleoside and nucleotide analogs; CHB, chronic hepatitis B

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addition, OMT accelerated the production of interferon- γ (IFN- γ) in a dose-dependent manner in CD4⁺ T cells. Our findings demonstrate the beneficial effects of OMT on the enhancement of immunological function and in the control of HBV antigens. The findings suggest this drug to be a good antiviral therapeutic candidate for the treatment of HBV infection.

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1. Introduction

Hepatitis B virus (HBV) infection is an urgent social problem with more than 350 million infected people worldwide¹. Consequences of HBV infection include a high risk for development of liver cirrhosis and/or hepatocellular carcinoma, which lead to high mortality². Nucleoside and nucleotide analogs (NAs), such as lamivudine, adefovir dipivoxil, and entecavir (ETV), are HBV reverse transcriptase inhibitors which potently decrease serum HBV DNA titer in patients³ and have been widely used in clinical treatment. Although the treatment of chronic hepatitis B (CHB) with NAs aims at inhibiting the HBV DNA reverse transcriptase polymerase, oral NAs do not exert a direct effect on hepatitis B surface antigen (HBsAg) transcription and translation⁴. Interferon- α remains an attractive treatment option for the HBsAg clearance in CHB^{5,6}. However, the unpleasant side effects and high expense of interferon- α in long-term therapy limit the use of this treatment⁷.

Traditional Chinese medicines (TCMs) have been widely used to treat chronic liver diseases for years^{8,9}. TCMs exert antiviral activity similar to that produced by interferon- α for the treatment of CHB, as demonstrated by the loss of serum hepatitis B e antigen (HBeAg) and HBV DNA¹⁰. Oxymatrine (OMT), a matrine-type alkaloid extracted from the Chinese herb *Sophora tonkinensis* Gagnep., has been shown to possess a wide range of pharmacological activity, including antiviral, immunoregulatory and inflammatory effects^{11,12}. According to recent studies, OMT inhibited the replication of HBV and the expression of HBsAg and HBeAg in HepG2.2.15 cells and in transgenic mice^{13,14}. The HBV transgenic mice demonstrated limited production and expression of HBV antigens, but were enitally tolerant to HBV antigens^{15,16}. Due to the limited availability of appropriate experiment models, earlier studies failed to detect OMT-induced immune responses to HBV infection in normal subjects.

To overcome the shortcomings of the existing models, an HBV persistence murine model was established by a single hydrodynamic injection of a replication-competent HBV DNA, pAAV/HBV1.2¹⁷. After the injection, HBV carrier mice expressed HBV-associated antigen and high level of serum HBV DNA, whereas the level of serum alanine aminotransferase is normal with no significant hepatic inflammation. The characteristics of this mouse model for HBV persistence are analogous to those of human CHB in the immune tolerant stage¹⁸.

In view of these advantages, the model was presently used to investigate the potency of OMT and its mechanism leading to HBV clearance. In this study, ETV and three different doses of OMT were concurrently administered and compared for the treatment of HBV. The efficacies of ETV and OMT were

evaluated by the levels of HBV DNA, HBsAg, HBeAg and hepatitis B core antigen (HBcAg). Subsequent experiments were conducted to evaluate the immune regulatory function of OMT by serum ELISA and flow cytometry.

2. Materials and methods

2.1. HBV plasmid

The plasmid (pAAV/HBV1.2), which contains the coding sequence for HBV genotype A, for the hydrodynamic injection was obtained from P.J. Chen (National Taiwan University, Taipei).

2.2. Mice and induction of HBV infection

C57BL/6 mice, weighting approximately 16–18 g, were obtained from Spfanimals Biotechnology Co., Ltd. (Certication number: SCXK-JING 2011-0004, Beijing, China). All of them received humane care according to the National Institutes of Health Guidelines for Animal Care and the Guidelines of the Scripps. Six micrograms of HBV plasmid DNA were injected into each mouse tail vein in a volume of saline equivalent to 10% of the mouse body weight. The total volume was delivered within 5–8 s.

2.3. Animal experiment design and drug treatment

Mice were tested for serum HBeAg at 1 week post-injection and divided into groups according to titers of HBeAg then arranged from the highest titers to the lowest. Animals were randomly divided into five groups with high to low titers in each group. The effects of three doses of OMT (2.2, 6.7 and 20 mg/kg) were studied. OMT was dissolved in saline and intraperitoneally injected once a day. ETV was administrated orally daily with the dose of 0.1 mg/kg according to the manufacturer instructions. The drugs were delivered for 6 weeks. The model group was injected with saline. A control group was treated with saline without plasmid transfection. OMT was purchased from Undersun Biomedtech Co., Ltd. (Xian, Shanxi, China). Entecavir baraclude was acquired from Sino-Squibb pharmaceutical Co., Ltd. (Shanghai, Beijing).

2.4. Detection of serum antigen and transaminase

Blood samples of mice were collected from the orbital vein after 1-week induction of plasmid injection and following 1, 3 and 6 weeks of treatment. Serum (5-fold dilution) levels of HBsAg and HBeAg were determined by the Chemiluminescence

Immunoassay system kits from Tigsun Diagnostics Co., Ltd. (Beijing, China). Transaminases were measured with test reagents provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All procedures were performed according to kit instructions.

2.5. Detection of serum HBV DNA by quantitative polymerase chain reaction

Serum samples were collected at indicated time points after the injection of pAAV/HBV 1.2. Each sample was pretreated with 2 units of DNase I (NEB) at 37 °C overnight and detected for HBV DNA by qPCR using a quantitative PCR Diagnostic kit for HBV DNA (Sino-MDgene Technology Co., Ltd., Beijing, China) and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

2.6. Serum ELISA

Following 6 weeks of treatment, serum was isolated from blood at 3500 rpm (LTDHC-3018R, Anhui Zhongke Scientific Instrument Co., Ltd, Anhui, China) for 10 min. IFN- γ and TNF- α were determined by the ELISA Kit (EXCELL Bio Co., Ltd., Shanghai, China). All the experiments were performed according to the manufacturer's instructions.

2.7. Flow cytometry

After 6 weeks of treatment, the whole spleen was taken and washed with cold PBS. Lymphocytes were isolated with lymphocyte separation medium after disruption with 200- μ m nylon net filters. The separation medium and fluorochrome-conjugated antibodies against CD3e (PerCP/cy5.5), CD8a (PE/Cy7) and CD4 (FITC) were obtained from Dakewe Biotech Co., Ltd. (Shenzhen, China). IFN- γ (FITC), IL-4 (PE), pyromellitic acid (PMA), ionomycin and monensin were purchased from BD Biosciences (New York, USA). For determination of cytokine, 1×10^6 lymphocytes were incubated with 1640 (contains 10% FBS) and stimulated with PMA (100 ng/mL) plus 1 μ g/mL of ionomycin, and 1 μ g/mL monensin at the same time. Five hours later, the cells were collected and stained with antibodies. After being stained the cells were fixed using 1% paraformaldehyde. All the samples were analyzed using FlowJo 7.6.1.

2.8. Hematoxylin and eosin and immunohistochemistry

Paraformaldehyde-fixed samples were embedded in paraffin, cut into 5 μ m-thick sections and mounted on a slide. Then, the sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry, slides were stained at 4 °C overnight with primary antibodies with HBcAg (rabbit anti-HBcAg, 1:200; Goodbio Technology, China). Then the slides were stained for 30 min with a secondary antibody (goat anti-rabbit HRP). We quantified the number of HBcAg-positive hepatocytes using image processing software (Image J, NIH, USA) on five random non-overlapping fields for each sample.

2.9. Statistical analysis

The data were analyzed with the SPSS software program (version 22.0, Chicago, IL, USA). One-way analysis of variance (ANOVA) with the *post hoc* test for parametric comparisons, or Kruskal–Wallis analysis of variance followed by the *post hoc* Dunn test for nonparametric comparisons were used for the evaluation of significant differences.

Normally distributed variables were expressed as mean \pm SD and abnormally distributed variables were expressed as median [25th and 75th percentile]. The differences were considered to be statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

3. Results

3.1. OMT promoted the clearance of serum HBV DNA

To investigate the efficacy of OMT in treating HBV infection, we used the immunocompetent mouse model which was established by hydrodynamical injection of pAAV/HBV1.2 to establish the chronic

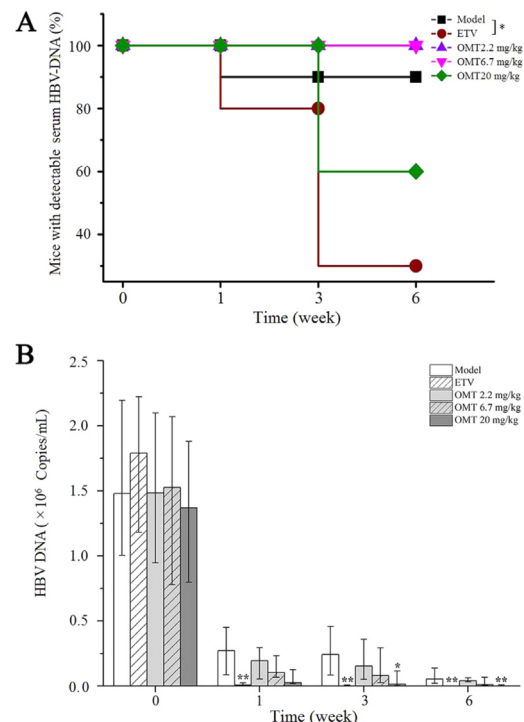


Figure 1 Oxymatrine promotes serum HBV DNA clearance. (A) Proportion of animals whose serum HBV-DNA can be detected before and after treatment of saline (model group), entecavir (ETV group), or three doses of oxymatrine (OMT groups) for 1, 3, and 6 weeks ($n = 10$). Log-rank Mantel-cox test was used. The limit of detection of serum HBV DNA was 300 copies/mL. (B) Serum HBV DNA levels in C57BL/6 mice in different groups ($n = 10$). Data are presented as median [25th and 75th percentile]. * $P < 0.05$, ** $P < 0.01$ compared with model group.

HBV infection. After injection, almost all subjects expressed high levels of HBV DNA in sera, whereas the control group (healthy animals) did not (data not shown). The level of serum HBV DNA was monitored regularly in the HBV-treated subjects. OMT was administrated intraperitoneally at the doses of 2.2, 6.7 and 20 mg/kg daily for 6 weeks. As shown in Fig. 1A, 90% of mice receiving saline exhibited detectable HBV DNA in serum throughout the experiment. ETV showed prominent inhibitory effects on viral replication from the first week; the percentage of mice with detectable HBV DNA were only 20% by the last week (Fig. 1A; $P < 0.05$). At the same time, OMT (20 mg/kg) tended to control the HBV infection, since the percentage of mice with detectable HBV DNA copies was 60% by the last week. To further characterize the viral titers, we also quantified the HBV DNA copies in the hydrodynamically injected mice using real-time PCR. ETV showed tremendous suppression effect on HBV infection in all the indicated time point (Fig. 1B; $P < 0.01$), and OMT at 20 mg/kg also reduced the HBV titers compared with the model group that still carried relatively high level of DNA copies at the 3rd and 6th week ($P < 0.05$, $P < 0.01$, respectively). However, OMT at 2.2 mg/kg and 6.7 mg/kg had no effect even with long-term administration. Thus, OMT exhibited a relatively lower efficacy on the reduction of serum HBV DNA compared to ETV treatment.

3.2. OMT reduced the expression of serum HBV antigen

Although ETV was able to reduce the level of serum HBV DNA in transfected mice, it had very limited impact on serum HBsAg (Fig. 2A). Before treatment, each group displayed nearly approximate levels of HBsAg and HBeAg. After the first week, OMT at 20 mg/kg showed a greater effect on the reduction of HBsAg compared with the model group (Fig. 2A; $P < 0.01$). In addition, prolonged therapy (3 and 6 weeks) further decreased the level of serum HBsAg as compared with the first week ($P < 0.01$ both). However, the carrier mice treated with low and medium dose of OMT (2.2 and 6.7 mg/kg) and oral ETV unremarkably eliminated the level of HBsAg. The results show that OMT at 20 mg/kg significantly reduced the level of HBsAg while ETV showed no preferential inhibition on HBsAg compared with the model group. To further investigate the effect of OMT, the level of HBeAg was determined in the experiment (Fig. 2B). The HBeAg was significantly decreased in the mice treated with OMT at 20 mg/kg compared with the model group at the first week ($P < 0.01$). After treatment for 3 weeks, ETV and OMT (20 mg/kg) caused significant decline in the induction of HBeAg ($P < 0.05$ and $P < 0.01$, respectively). In addition, after 6 weeks of treatment, ETV and OMT at 6.7 mg/kg and 20 mg/kg showed obvious reductions of HBeAg ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively). Further comparative analysis revealed that OMT at 20 mg/kg was more efficient than ETV on the reduction of HBeAg at the last week of treatment ($P < 0.05$). The high efficiency of OMT in reducing HBsAg and HBeAg was the major feature distinguishing this drug from ETV.

3.3. OMT influenced the persistence of intrahepatic HBcAg

Liver tissue was collected after 6-week treatment to further characterize the expression of HBcAg through immunohistochemical staining. We observed that all the mice receiving pAAV/HBV1.2 expressed HBcAg (Fig. 3). Thus, both ETV and OMT (at 6.7 and 20 mg/kg) were all sufficient to decrease the count of HBcAg-

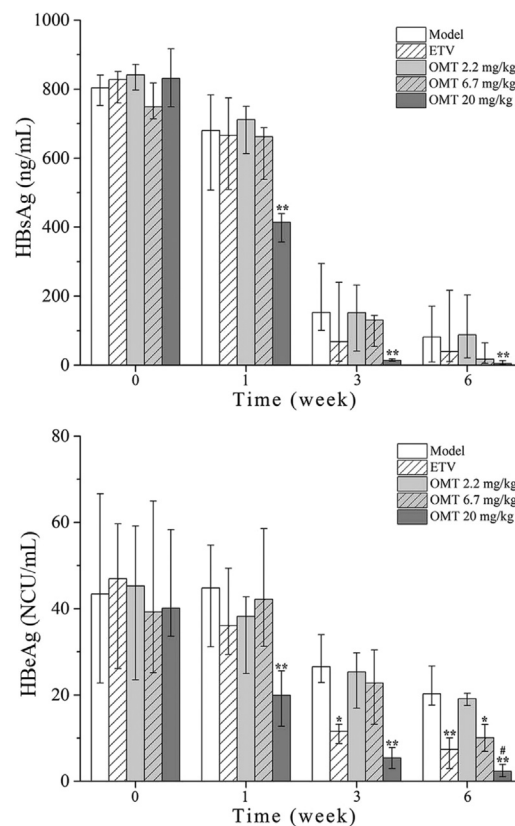


Figure 2 Oxymatrine accelerates the clearance of HBsAg and HBeAg. The levels of serum HBsAg (A) and HBeAg (B) in infected mice were measured. Data are presented as median [25th and 75th percentile]. * $P < 0.05$, ** $P < 0.01$ compared with model group; $P < 0.05$ compared with entecavir group (ETV); $n = 10$.

expressing hepatocytes compared with the model group ($P < 0.01$). Notably, OMT at 20 mg/kg inhibited HBeAg more remarkably than ETV did (Fig. 3F, $P < 0.01$). Collectively, the data indicate that OMT efficiently suppressed the expression of HBV antigens.

3.4. OMT promoted the expression of IFN- γ

Previous research has shown that T-cell-mediated responses are essential in the initial control of HBV infection, especially CD4 T cell and cytokines IFN- γ and TNF- α ¹⁹. Other studies have demonstrated the functions of T-helper cells: Th1 cells (cellular immunity) lead the attack against intracellular pathogen, a pathway which is heavily reliant on IFN- γ . Th2 cells (humoral immunity) are believed to up-regulate antibody production to fight extracellular organisms, a pathway heavily dependent upon IL-4. Both the cellular and humoral immune responses play roles in controlling HBV^{20,21}. Based on these observations, we examined the related cytokines in serum by ELISA. OMT at 6.7 and 20 mg/kg promoted the expression of IFN- γ (Fig. 4A; $P < 0.05$, $P < 0.01$, respectively), but displayed no influence on TNF- α (Fig. 4B). To further delineate the efficiency of OMT, we investigated the proportions of CD3⁺ CD4⁺ T cells and CD3⁺ CD8⁺ T cells in spleen lymphocytes by flow cytometry; results no differences in each group (Fig. 4C and D). To further clarify the importance of Th1 and Th2 cells in HBV control and

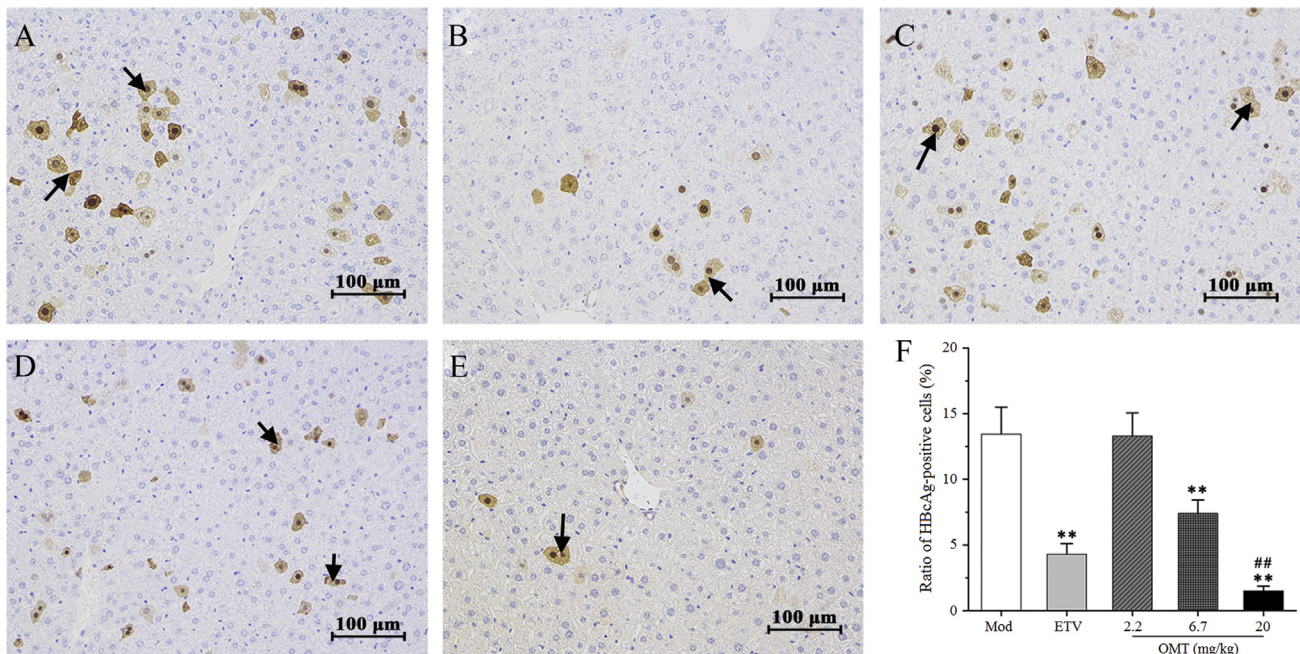


Figure 3 Oxymatrine influences the persistence of intrahepatic HBcAg. Immunohistochemistry staining (brown HBcAg-expressing hepatocytes) of liver sections from infected mice: (A) Model; (B) entecavir (ETV); (C)–(E) intraperitoneal injection of OMT at 2.2, 6.7 and 20 mg/kg, respectively. (F) Statistical analysis from each visual field (200 \times magnification). ** $P < 0.01$ compared with the model group; $p < 0.01$ compared with the ETV group; $n = 6$.

clearance, CD3⁺ CD4⁺ T cells were gated and examined by intracellular staining of IFN- γ and IL-4. The result indicated that after HBV infection the expression of IFN- γ produced by CD4⁺ T and CD8⁺ T cells were significantly restrained (Fig. 4E and F; $P < 0.01$, $P < 0.05$) while the expression of CD4⁺ T cells producing IL-4 were enhanced (Fig. 4G; $P < 0.05$). After OMT treatment, the

expression of CD4⁺ T cells producing IFN- γ increased in a dose-dependent manner (Figs. 4E and 5; $P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) but this treatment had no influence on IL-4 (Fig. 4G). Meanwhile, only OMT at 20 mg/kg exhibited an enhancement of CD8⁺ T cells producing IFN- γ (Fig. 4F; $P < 0.05$). Consequently

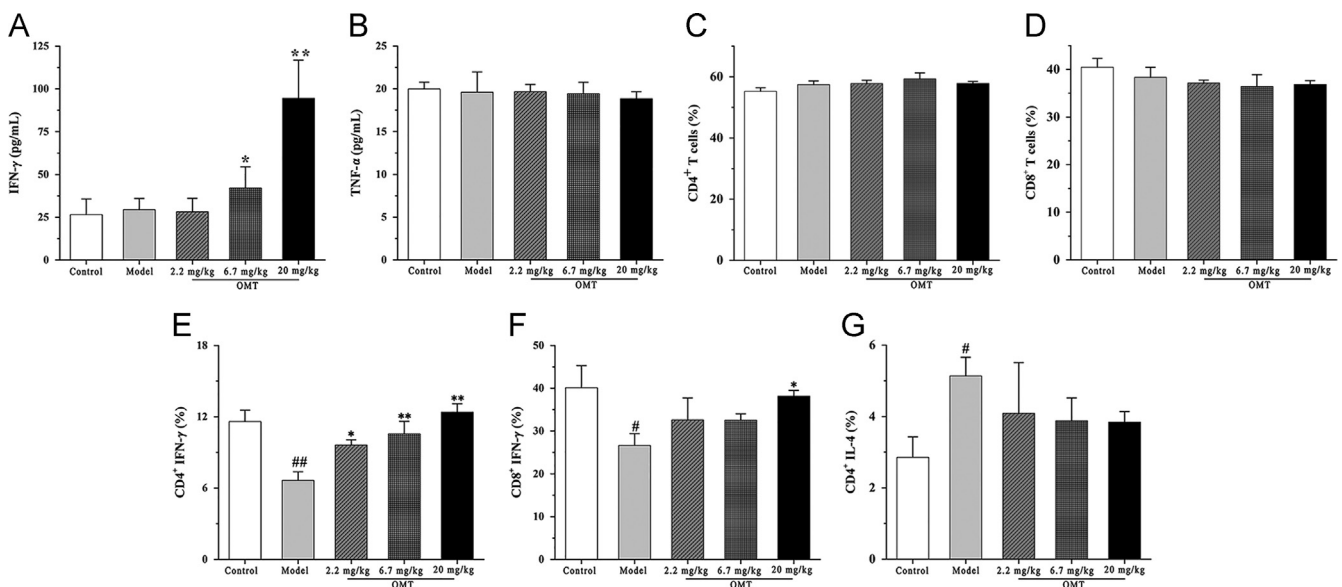


Figure 4 Cytokine revolved in HBV clearance in serum and spleen lymphocyte. Quantification of serum cytokine levels of IFN- γ (A) and TNF- α (B). The whole spleens were taken after 6-week treatment. Flow cytometry analysis showing the proportion of CD4⁺ and CD8⁺ T cells and the proportion of IFN- γ produced by CD4⁺ and CD8⁺ T cells in spleen lymphocytes (C)–(F) and IL-4 produced by CD4⁺ T cells (G). * $P < 0.05$, ** $P < 0.01$ compared with model group; $P < 0.05$, $P < 0.01$ compared with the control group; $n = 6$.

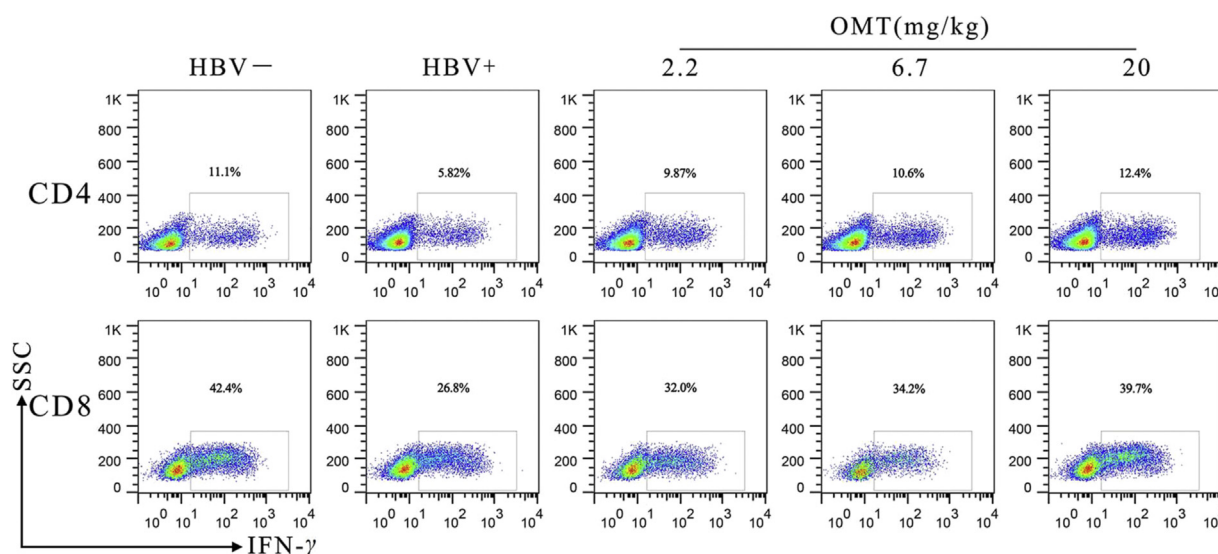


Figure 5 IFN- γ is promoted in splenic T cells. Quantification of IFN- γ -positive cells by flow cytometry in CD4⁺ and CD8⁺ T cells from infected mice; $n = 6$.

the efficacy of OMT on HBV clearance is very likely to be related to the large numbers of CD4⁺ T cells producing IFN- γ .

3.5. No liver injury was observed after OMT treatment

The first stage in HBV infection is characterized by a period of immune tolerance. During this phase, there is active viral replication, but no symptoms and no significant increase in serum alanine aminotransferase.²² The improvement of IFN- γ indicated the immunological enhancement action of OMT. Therefore, we investigated whether OMT could cause subsequent liver damage with long term administration. H&E examination and serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) demonstrated that both OMT and oral ETV did not produce liver damage (Fig. 6A–H). Collectively, these data indicate that no overt liver damage was observed either histologically or biochemically.

4. Discussion

HBV is an enveloped and double-strand DNA virus, and the persistence of HBV covalently closed circular DNA in hepatocytes will lead to chronic hepatitis B infection²³. HBV is not directly cytopathic and liver injury appears to be mostly caused by repeated attempts of the host's immune responses to control the infection²⁴. Ineffective immune response against HBV may result in persistent virus replications, then lead to CHB²⁵. Herein, the strategies of treating HBV infection generally aim to control HBV through direct viral suppression or by restoring antiviral host immunity^{26,27}.

Although HBV infection restricts host range and only humans and chimpanzees are susceptible to HBV infection, several HBV infection models have been created²⁸. HBV transgenic mice have been used to evaluate numerous drugs for HBV infection; however, the central tolerance induced by the transgenic gene products made the study of immune therapy difficult²⁹. Chimeric mice can be infected with human hepatotropic viruses, but adaptive immune responses are absent (B, T, and NK cells) and HBV cannot be cleared in this model^{30,31}.

In the present study, we used a hydrodynamics-based immunocompetent mouse model which was able to mimic multiple features of HBV natural infection observed in humans, including age-dependent chronicity, genetic variations influencing the outcomes of HBV infection, and the possible interactions between them³². After the injection, the average titer of serum HBV DNA in each animal reached about 2.0×10^6 copies and the mice expressed HBsAg, HBeAg and HBcAg. In this study, administration of ETV resulted in the rapid control of HBV infection, while OMT exhibited a relatively lower efficacy on the reduction of serum HBV DNA. Although ETV caused a profound suppression of virus production, it had no influence on serum HBsAg and very little impact on serum HBeAg and intrahepatic HBcAg. Conversely, treatment of 20 mg/kg OMT was sufficient to decrease the levels of serum HBsAg and HBeAg, and intrahepatic HBcAg. Consistent with previous clinical reports^{33–36}, OMT accelerated the rate that HBeAg and HBV DNA converted to negative, confirming the inhibition effect of OMT on HBV infection observed in our investigation.

Cytokines play a key role in the regulation of the immune response and control the infection. Th1-associated cytokines are well known for playing a central role in HBV clearance and also contribute to influence the pathogenesis of liver disease. Th2 cells are related to proliferation and differentiation of B cells, as well as antibody generation²⁵. IFN- γ has been demonstrated to be important as a mediator for antiviral activity, and it may activate a variety of IFN-inducible genes, trigger intracellular antiviral pathways and inhibit the HBV replication cycle both transcriptionally and post-transcriptionally³⁷. Induction of serum IFN- γ levels in the treatment of CHB patients led to virological control and HBeAg seroconversion³⁸. In the present study, OMT (6.7 and 20 mg/kg) was sufficient to promote the expression of IFN- γ both in peripheral blood and spleen lymphocytes. The data indicate that OMT activated innate immunity to induce IFN- γ production and inhibited the replication of HBV-infected cells, thus directly reducing viral load. Several groups have indicated that both CD4⁺ and CD8⁺ T cells assist in the control of HBV in acute models of infection^{39,40}. Our model may be more representative of a chronic infection model in which infected CD4⁺ T cell depleting

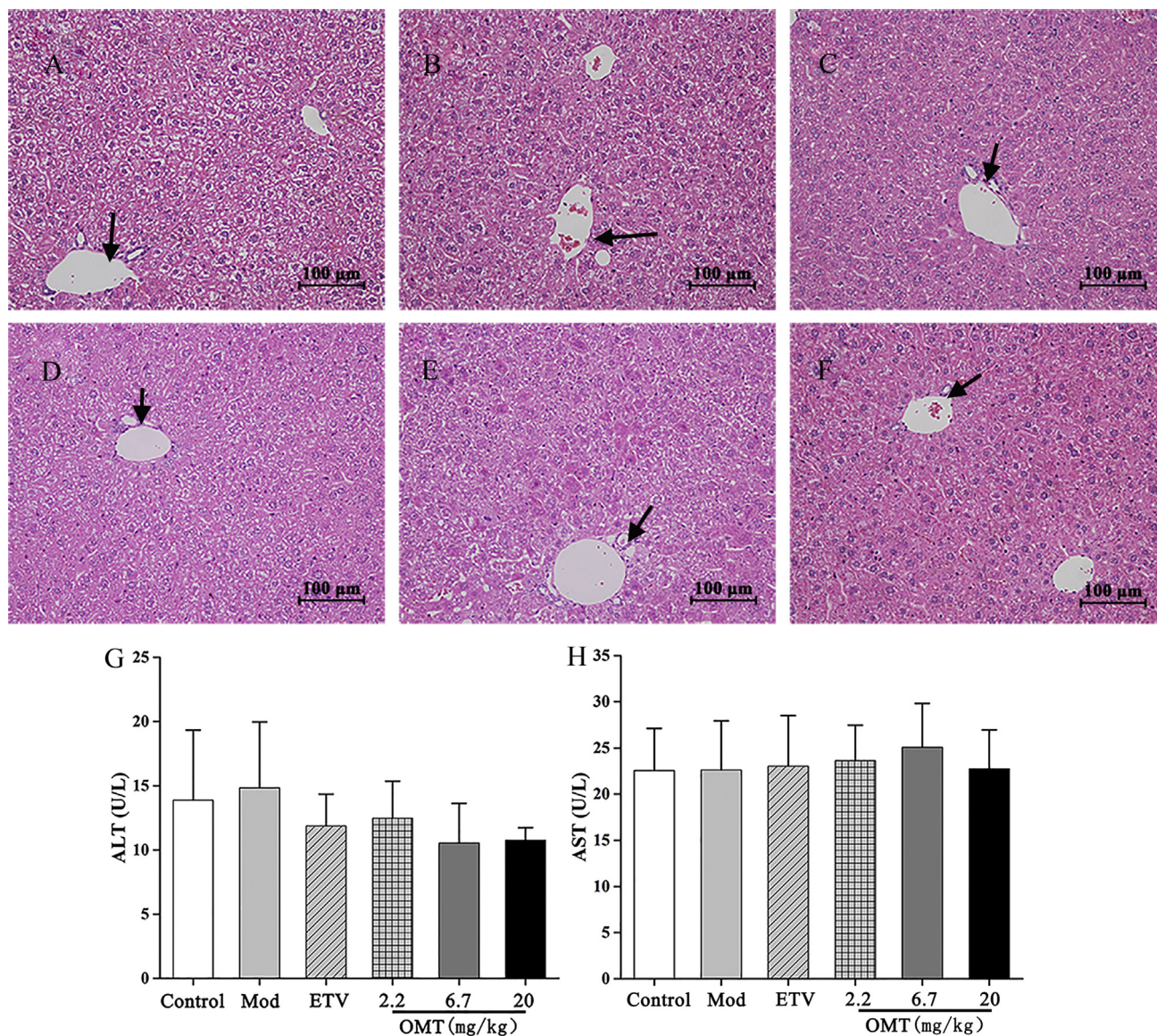


Figure 6 Liver H&E staining and serum level of ALT and AST. H&E staining (200 × magnification, $n = 6$): (A) Control; (B) Model; (C) Entecavir treatment (ETV); (D)–(F) Oxymatrine treatment at 2.2, 6.7 and 20 mg/kg, respectively. (G) and (H) Serum levels of ALT and AST; $n = 10$.

carrier mice were unable to control viremia while $CD8^+$ T cell depletion did not impair HBV control¹⁹.

Accordingly, our research suggests that the efficacy of the anti-viral activity of OMT is due to activation of innate immunity to induce production of $IFN-\gamma$ in $CD4^+$ T cells and inhibition of the replication of HBV. Due to the effect of immunologic enhancement and the efficacy of HBV antigen reduction, OMT seems to be a good antiviral therapeutic candidate to treat HBV infection. However, the mechanism of OMT on promoting $IFN-\gamma$ production and HBV antigen clearance requires further study.

5. Conclusions

In the present study, as an appropriate model for human HBV infection, mice were quickly hydrodynamically injected with 6 μ g of HBV plasmid DNA into tail veins in a volume of saline equivalent to 10% of the mouse body weight. In this model, ETV

was able to reduce the level of serum HBV DNA in infected mice, while it had very limited impact on serum HBsAg. OMT, administrated intraperitoneally, was beneficial for the control of HBV infection and it produced obvious reduction in HBsAg, HBeAg and HBcAg. Subsequent experiments indicated that the efficacy of OMT on HBV clearance was mediated by $IFN-\gamma$ produced in $CD4^+$ T cells. In conclusion, the immunologic mechanism and effect of OMT on the control of HBV were investigated in an immunocompetent mouse model. Due to the high efficiency on the reduction of HBV antigen levels, OMT appears to be a good antiviral therapeutic candidate to treat HBV infection.

Acknowledgments

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References

- Liaw YF, Chu CM. Hepatitis B virus infection. *Lancet* 2009;**373**:582–92.
- Lupberger J, Hildt E. Hepatitis B virus-induced oncogenesis. *World J Gastroenterol* 2007;**13**:74–81.
- Dienstag JL. Benefits and risks of nucleoside analog therapy for hepatitis B. *Hepatology* 2009;**49**:S112–21.
- Chevaliez S, Hézode C, Bahrami S, Grare M, Pawlotsky JM. Long-term hepatitis B surface antigen (HBsAg) kinetics during nucleoside/nucleotide analogue therapy: finite treatment duration unlikely. *J Hepatol* 2013;**58**:676–83.
- Cornberg M, Höner Zu Siederdisen C. HBsAg seroclearance with NUCs: rare but important. *Gut* 2014;**63**:1208–9.
- Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN- α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012;**122**:529–37.
- Perrillo R. Benefits and risks of interferon therapy for hepatitis B. *Hepatology* 2009;**49**:S103–11.
- Wang BE. Treatment of chronic liver diseases with traditional Chinese medicine. *J Gastroenterol Hepatol* 2000;**15** Suppl:E67–70.
- Luk JM, Wang X, Liu P, Wong KF, Chan KL, Tong Y, et al. Traditional Chinese herbal medicines for treatment of liver fibrosis and cancer: from laboratory discovery to clinical evaluation. *Liver Int* 2007;**27**:879–90.
- Zhang L, Wang G, Hou W, Li P, Dulin A, Bonkovsky HL. Contemporary clinical research of traditional Chinese medicines for chronic hepatitis B in China: an analytical review. *Hepatology* 2010;**51**:690–8.
- Zheng P, Niu FL, Liu WZ, Shi Y, Lu LG. Anti-inflammatory mechanism of oxymatrine in dextran sulfate sodium-induced colitis of rats. *World J Gastroenterol* 2005;**11**:4912–5.
- Zhang Y, Wang S, Li Y, Xiao Z, Hu Z, Zhang J. Sophocarpine and matrine inhibit the production of TNF- α and IL-6 in murine macrophages and prevent cachexia-related symptoms induced by colon26 adenocarcinoma in mice. *Int Immunopharmacol* 2008;**8**:1767–72.
- Lu LG, Zeng MD, Mao YM, Fang JY, Song YL, Shen ZH, et al. Inhibitory effect of oxymatrine on serum hepatitis B virus DNA in HBV transgenic mice. *World J Gastroenterol* 2004;**10**:1176–9.
- Lin M, Yang LY, Li WY, Peng YP, Zheng JK. Inhibition of the replication of hepatitis B virus *in vitro* by oxymatrine. *J Int Med Res* 2009;**37**:1411–9.
- Tzeng HT, Hsu PN, Chen PJ. Immunocompetent nontransgenic mouse models for studying hepatitis B virus immune responses. *J Gastroenterol Hepatol* 2013;**28** Suppl 1:116–9.
- Dembek C, Protzer U. Mouse models for therapeutic vaccination against hepatitis B virus. *Med Microbiol Immunol* 2015;**204**:95–102.
- Liang SQ, Du J, Yan H, Zhou QQ, Zhou Y, Yuan ZN, et al. A mouse model for studying the clearance of hepatitis B virus *in vivo* using a luciferase reporter. *PLoS One* 2013;**8**:e60005.
- Huang LR, Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. *Proc Natl Acad Sci U S A* 2006;**103**:17862–7.
- Ebert G, Preston S, Allison C, Cooney J, Toe JG, Stutz MD, et al. Cellular inhibitor of apoptosis proteins prevent clearance of hepatitis B virus. *Proc Natl Acad Sci U S A* 2015;**112**:5797–802.
- Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* 2003;**8**:223–46.
- Bertoletti A, Gehring AJ. The immune response during hepatitis B virus infection. *J Gen Virol* 2006;**87**:1439–49.
- Wright TL. Introduction to chronic hepatitis B infection. *Am J Gastroenterol* 2006;**101** Suppl 1:S1–6.
- Beck J, Nassal M. Hepatitis B virus replication. *World J Gastroenterol* 2007;**13**:48–64.
- Dandri M, Locarnini S. New insight in the pathobiology of hepatitis B virus infection. *Gut* 2012;**61** Suppl 1:i6–17.
- Li X, Liu X, Tian L, Chen Y. Cytokine-mediated immunopathogenesis of Hepatitis B virus infections. *Clin Rev Allergy Immunol* 2016;**50**:41–54.
- Bertoletti A, Rivino L. Hepatitis B: future curative strategies. *Curr Opin Infect Dis* 2014;**27**:528–34.
- Jiang Y, Ma Z, Xin G, Yan H, Li W, Xu H, et al. Th1 and Th2 immune response in chronic hepatitis B patients during a long-term treatment with adefovir dipivoxil. *Mediat Inflamm* 2010;**2010**:143026.
- Dandri M, Lütgehetmann M, Petersen J. Experimental models and therapeutic approaches for HBV. *Semin Immunopathol* 2013;**35**:7–21.
- Yang D, Liu L, Zhu D, Peng H, Su L, Fu YX, et al. A mouse model for HBV immunotolerance and immunotherapy. *Cell Mol Immunol* 2014;**11**:71–8.
- Dandri M, Petersen J. Chimeric mouse model of hepatitis B virus infection. *J Hepatol* 2012;**56**:493–5.
- Allweiss L, Volz T, Lütgehetmann M, Giersch K, Bornscheuer T, Lohse AW, et al. Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration. *J Hepatol* 2014;**60**:500–7.
- Chou HH, Chien WH, Wu LL, Cheng CH, Chung CH, Horng JH, et al. Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota. *Proc Natl Acad Sci U S A* 2015;**112**:2175–80.
- Lu LG, Zeng MD, Mao YM, Li JQ, Wan MB, Li CZ, et al. Oxymatrine therapy for chronic hepatitis B: a randomized double-blind and placebo-controlled multi-center trial. *World J Gastroenterol* 2003;**9**:2480–3.
- Chen XS, Wang GJ, Cai X, Yu HY, Hu YP. Inhibition of hepatitis B virus by oxymatrine *in vivo*. *World J Gastroenterol* 2001;**7**:49–52.
- Lu LG, Zeng MD, Mao YM, Wan MB, Li CZ, Chen CW, et al. Oxymatrine in the treatment of chronic hepatitis B for one year: a multicenter random double-blind placebo-controlled trial. *Chin J Hepatol* 2004;**12**:597–600.
- Yu Y, Si C, Zeng Z, Wang Q, Zhou X, Zhang Q, et al. A clinical trial of oxymatrine in treating chronic viral hepatitis type B. *Chin J Intern Med* 2001;**40**:843–6.
- Uprichard SL, Wieland SF, Althage A, Chisari FV. Transcriptional and posttranscriptional control of hepatitis B virus gene expression. *Proc Natl Acad Sci U S A* 2003;**100**:1310–5.
- Chokshi S, Cooksley H, Riva A, Phillips S, Williams R, Gaggari A, et al. Identification of serum cytokine profiles associated with HBsAg seroconversion following antiviral treatment interruption. *Viral Immunol* 2014;**27**:235–44.
- Yang PL, Althage A, Chung J, Maier H, Wieland S, Isogawa M, et al. Immune effectors required for hepatitis B virus clearance. *Proc Natl Acad Sci U S A* 2010;**107**:798–802.
- Thimme R, Wieland S, Steiger C, Ghayeb J, Reimann KA, Purcell RH, et al. CD8⁺ T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003;**77**:68–76.