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• VIRAL HEPATITIS •

# Inhibitory effect of oxymatrine on serum hepatitis B virus DNA in HBV transgenic mice

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## Abstract

**AIM:** To study the inhibitory effect of oxymatrine on serum hepatitis B virus (HBV) DNA in HBV transgenic mice.

**METHODS:** HBV transgenic mice model was established by microinjection, and identified by HBV DNA integration and replication. Transgenic mice with replicating HBV were divided into 3 groups, and injected with normal saline (group A, $n$=9), 50 mg/kg (group B, $n$=8) and 100 mg/kg (group C, $n$=9) oxymatrine intraperitoneally once a day for 30 d, respectively. Quantitation of serum HBV DNA in HBV transgenic mice was performed by competitive polymerase chain reaction (PCR) in combination with DNA hybridization quantitative detection technique before and after treatment.

**RESULTS:** Compared with pre-treatment, the serum HBV DNA in group A (F=1.04, $P$=0.9612) and group B (F=1.13, $P$=0.8739) had no changes after treatment. However, in group C serum HBV DNA was significantly decreased (F=13.97, $P$=0.0012). The serum HBV DNA after treatment was lower in group C than in groups B and A (F=8.65, $P$=0.0068; F=12.35, $P$=0.0018; respectively). The serum HBV DNA after treatment was lower in group B than in group A, but there was no statistical significance (F=1.43, $P$=0.652).

**CONCLUSION:** Oxymatrine has inhibitory effects on serum HBV DNA in HBV transgenic mice.

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular cancer and accounts for 1 million deaths annually. Information on the virus load and the replicative activity of HBV is of paramount importance in the management of patients with chronic HBV infection, because of recent advances in medications which can effectively suppress HBV replication by using nucleoside analogues, interferon and other drugs[1-8]. Serological parameters include the levels of hepatitis B e antigen (HbeAg), DNA polymerase, HBV DNA measured by qualitative methods such as PCR or semi-quantitative dot blot hybridization, and HBV DNA quantitation by solution hybridization. The direct and quantitative nature of HBV DNA quantitation by solution hybridization makes it a useful clinical test to monitor serially the efficacy of antiviral therapy[9-15].

The aim of chronic hepatitis B treatment is to control infectivity, eradicate the virus and prevent the development of cirrhosis. However, permanent loss of HBeAg and HBV DNA is unusual and HBsAg persistently presents. For many years, alpha interferon was the only approved therapy for chronic HBV infection in most countries around the world. Interferon is effective in only 30-40% patients. It must be given by injection and is frequently associated with fever and flu-like symptoms[1-3]. Recently, lamivudine was approved for the treatment of chronic HBV infection in many regions of the world[1]. Although convenient and well-tolerated, lamivudine's efficacy is similar to that of interferon and prolonged administration of lamivudine is associated with development of resistance[6]. New agents, such as adefovir dipivoxil, offer promising effects when used either alone or in combination with lamivudine in the treatment of individuals who are 'treatment naï ve' or have developed lamivudine resistance[3,6]. Up to now, there is no specific therapy available for chronic hepatitis B. Therefore, the search continues for more efficacious treatments.

Many basic and clinical studies have revealed that oxymatrine has anti-hepatitis B virus effect, but the mechanism is unclear[16-20]. The hosts of HBV are very scarce, and *ex vivo* culture of HBV is very difficult, thus limiting the study on HBV and its related diseases. Since 1985, a variety of HBV transgenic mice have come out in succession, providing a new approach to study the mechanism and treatment methods of HBV and its related diseases[21-26]. Serum HBV DNA in transgenic mice was quantitated by competitive polymerase chain reaction (PCR) in combination with DNA hybridization technique in this study to assess the anti-HBV effect of oxymatrine, and to provide an important experimental basis for its clinical application.

## MATERIALS AND METHODS

Plasmid P1.0 HBV was donated by Professor Xiang-Fu Wu (Institute of Biochemistry, Chinese Academy of Sciences). The complete genome (3.2 kb) of adr-type HBV was cloned at the *BamH1* site in PUC18 vector. ICR mice aged 8 wk were bought from Animal Center of Yangzhou University, raised in depurated animal rooms. Transgenic HBV mice obtained from Dr. Cheng Yong (Yangzhou University, Jiangsu Province) were used.

### *Detection of HBV integration in transgenic mice*

Genomic DNA was extracted from the tails of HBV transgenic mice (about 1.0-1.5 cm) at age of 4 wk[25]. The sequences of PCR primers were 5' -AGAGTCTAGACTCGTGGTGGACTT-3' and 5' -TACGAACCACTGAACAAATGGCAC-3' . The amplified segment was in the region of 470 bp HBc DNA. PCR reaction mixture containing 5 μL 10×buffer (0.5 mol/L KCl, 0.1 mol/L

Tris-HCl pH8.3, 0.015 mol/L $MgCl_2$, 0.1 g/L glutin), 35 μL sterilized water, 5 μL 2 mmol/L dNTP, and 2 mL primers 1 and 2, was heated for 10 min at 95 ℃ to denature DNA. A 1 μL of Taq DNA polymerase was added to the mixture, which was overlaid with 50 μL of light mineral oil. The amplification reaction was run for 35 cycles as follows: denaturing at 93 ℃ for 60 s, annealing at 60 ℃ for 45 s, and extension at 72 ℃ for 1 min. A 5 μL amplification product was loaded into agarose gel for electrophoresis.

### *Identification of HBV expression in transgenic mice*

The experimental procedures followed the instructions in the kit of Amplicor HBV monitor (Roche Diagnostic Systems, Inc.). Blood from positive HBV transgenic mice was collected and the serum was separated. Internal control was added to standard and samples. PCR was performed on HBV DNA extracted from serum. The sequences of primers were 5'-GTTGCCCGTTTGTCCTCTAC-3', and 5'-biotin-GATGATGTGGTATTGGGGGC-3'. The reaction mixture was heated for 5 min at 94 ℃ to denature DNA, then amplification was carried out 30 cycles: denaturing at 94 ℃ for 30 s, annealing at 57 ℃ for 30 s, and extension at 72 ℃ for 45 s, plus a final extension at 72 ℃ for 5 min. The amplification product was separated by agarose gel electrophoresis, identified and quantitateded by hybridization.

### *Quantitation by enzyme linked hybridization in microwell plate*

The experiments were performed according to the instructions of the Amplicor HBV monitor kit.

The common probe (MXH2) was diluted with binding buffer. The sequence of the probe was 3'-AGCGTCCAGTCGTCGTTACAC-5' (500 nmol/L). A 50 μL of the diluted probe was added to each well in two DNA microwell plates and incubated at 37 ℃ for 2 h.

### *Pre-hybridization*

The solution in the well was discarded, 50 μL wild segment capture probe diluted by 1×hybridization solution was added to each well of one plate. The sequence of the probe was 5' -TCGCAGGTCAGCAGCATGTGACATCAACTACCAGCACG-3' (500 nmol/L). Fifty μL mutation primer diluted by 1×hybridization solution was added to each well of the other plate. The sequence of the probe was 5' -GTTGCCCGTTTGTC CTCTACATCACACAAAGATCTACGTCGACGCAGGACCATGCA AGACCT-3' (500 nmol/L). The plate was incubated at 55 ℃ for 60 min, and then the solution was discarded. The plate was dried and washed twice with solution I.

### *Hybridization*

The PCR products were diluted 10 times with distilled water, heated for 5 min at 100 ℃ to denature the DNA, then placed on ice immediately. One volume 2×hybridization solution was added to the product and 50 μL was added to the well. They were incubated at 48 ℃ for 60 min. The wells were washed three times with solution I at 48 ℃ for 3 min each. The solution was discarded and the plate was dried.

### *Signal assay*

A 50 μL 1:1 000 diluted streptavidin-ALP complex was added to each well and incubated at 37 ℃ for 20 min. They were then washed four times for 4 min at 37 ℃ with solution II, dried, and stained with the substrate (p-nitrooheno-sodium phosphate). Absorbance was read at 405 nm after 2 h.

### *Effect of oxymatrine on HBV transgenic mice*

Twenty-six transgenic mice aged 8-12 wk (either sexes) were divided into 3 groups, and injected with normal saline (group A, $n$=9), 50 mg/kg (group B, $n$=8) and 100 mg/kg (group C, $n$=9) oxymatrine intraperitoneally once a day for 30 d, respectively. Serum HBV DNA in HBV transgenic mice was detected by competitive polymerase chain reaction (PCR) in combination with DNA hybridization quantitative detection technique before and after treatment.

### *Statistical analysis*

All the data were analyzed by SAS software. ANOVA was used to evaluate the significance of the quantity of serum HBV DNA. $P$<0.05 was considered statistically significant.

## RESULTS

### *HBV DNA integration and replication in transgenic mice*

Among the 396 transgenic mice, 137 had HBV DNA integration (34.59%), and 26 had HBV replication in serum (6.56%).

The results of HBV DNA quantitation in transgenic mice before and after oxymatrine treatment are summarized in Table 1.

The results showed that there was no significant difference in HBV DNA quantity before treatment among the three groups. Compared with before treatment, there was no change in HBV DNA quantity in group A (F=1.04, $P$=0.9612). HBV DNA quantity was decreased in group B (F=1.13, $P$=0.8739), and was significantly decreased in group C (F=13.97, $P$=0.0012) after treatment. The level of HBV DNA was significantly lower in group C than in groups A (F=12.35, $P$=0.0018) and B (F=8.65, $P$=0.0068). HBV DNA quantity of group B after treatment was lower than that of group A, but there was no statistical difference (F=1.43, $P$=0.652).

## DISCUSSION

With the advent of embryo microinjection technology, it was clear that many questions related to HBV infection might be directly examined by introduction of a partial or complete copy of HBV genome into transgenic mice. Several lines of HBV-transgenic mice have been prepared by introducing either the full HBV genome, or a partial or selected portion of HBV genome into an inbred strain of mice. The successful estalishment of HBV-transgenic mice model and the expression of HBV gene products have shown that mice might be a host for the expression of HBV-related products. The characteristics of HBV infection in HBV transgenic mice have shown its potential utility as a model for study of anti-HBV drugs[22-24,27-35]. HBV transgenic mice were generated by microinjection, our results revealed that HBV genome was integrated in the genome of mice and expressed effectively. It proved that we could obtain mice bearing complete HBV genomes. In the 396 transgenic mice, 137 had HBV DNA integration (34.59%), and 26 had HBV replication in serum (6.56%). The existence of HBV DNA in serum demonstrated that there was HBV gene replication in mice[28,30,34,35].

**Table 1** Results of HBV DNA quantitation in transgenic mice before and after oxymatrine treatment (fg/mL) ($n$, mean±SD)

| Group | Group A | | Group B | | Group C | |
|---|---|---|---|---|---|---|
| Pre-treatment | 9 | 10 933.33±5 591.06 | 8 | 12 762.5±4 891.96 | 9 | 10 164.44±5 842.93 |
| Post-treatment | 9 | 10 864.44±5 492.81 | 8 | 5 898.375±4 597.09 | 9 | 2 426.33±1 563.12$^{b,d}$ |

$^b$$P$<0.01 *vs* before treatment, $^d$$P$<0.01 (*vs* after treatment in groups A and B).

Anti-HBV therapy of hepatitis B is still a difficult problem. Even the generally known drugs such as a-IFN and lamivudine have limitations such as low rate of HBV negativity, high price, side effects and virus variation. So it has become urgent to find new and effective anti--HBV drugs[1-3]. Oxymatrine has been found to be a kind of alkaloid extracted from a Chinese herb *Sophora alopecuraides L.*[20,36]. Basic and clinical researches have shown that oxymatrine has the following pharmacological effects: antivirus, protecting hepatocytes, antihepatic fibrosis, and immune regulation[37-41]. In particular, its inhibitory effect on hepatitis B virus (HBV) has attracted wide attention in recent years. Oxymatrine has been proven to have distinct anti-virus effects in the treatment of chronic hepatitis B (CHB)[16-20].

*In vivo* experiment indicated that oxymatrine could inhibit HBsAg and HBeAg secretion from HBV DNA transinfected cell strain 2.2.15. In a certain range, with increased concentration and reaction time, the inhibitory rate increased gradually. Given the same concentration and reaction time, the inhibitory rate on HBsAg appeared to be higher than that on HBeAg[42]. In an *in vivo* study on transgenic mice, the mice were injected intraperitoneally with 100 mg/kg, 200 mg/kg and 300 mg/kg oxymatrine everyday for 30 d. The amount of HBsAg and HBeAg decreased significantly compared with control group, and there was no difference among the treatment groups[19,42]. A clinical research suggested that when oxymatrine was applied to treat chronic hepatitis B, the normalization rates of serum ALT and TB, and the negative conversion rates of serum HBsAg and HBV DNA were similar to those with alpha interferon [42].

The amount of HBV DNA reflected the level of HBV replication directly. In this study we observed the effect of oxymatrine (50 mg/kg, 100 mg/kg) on HBV transgenic mice, and oxymatrine in both concentrations could inhibit the replication of HBV DNA. The results indicate that oxymatrine has inhibitory effects on the *in vivo* replication of HBV DNA. We still need to further explore how oxymatrine inhibits HBV DNA replication.

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