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BRIEF ARTICLE

Effect of oxymatrine on the replication cycle of hepatitis B virus *in vitro*

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Abstract

AIM: To determine the antiviral mechanism or target of oxymatrine against hepatitis B virus (HBV).

METHODS: HepG2.2.15 cells were incubated with culture medium containing 500 μg/mL of oxymatrine for 2 and 5 d. The surface antigen of HBV (HBsAg) and e antigen of HBV (HBeAg) in supernatant were determined by ELISA. HBV DNA in supernatant, and intracellular covalently closed circular DNA (cccDNA), relaxed circular DNA (rcDNA) and pregenomic RNA (pgRNA) were quantified by specific real-time polymerase chain reaction (PCR) or reverse transcription (RT)-PCR.

RESULTS: Treatment with oxymatrine for 2 d and 5 d reduced the production of HBV by the cell line, as indicated by the decline of HBsAg (22.67%, t = 5.439, P = 0.0322 and 22.39%, t = 5.376, P = 0.0329, respectively), HBeAg (55.34%, t = 9.859, P = 0.0101 and 43.97%, t = 14.080, P = 0.0050) and HBV DNA (40.75%, t = 4.570, P = 0.0447 and 75.32%, t = 14.460, P = 0.0047) in the supernatant. Intracellular cccDNA was also markedly reduced by 63.98% (t = 6.152, P = 0.0254) and 80.83% (t = 10.270, P = 0.0093), and intracellular rcDNA by 34.35% (t = 4.776, P = 0.0413) and 39.24% (t = 10.050, P = 0.0097). In contrast, intracellular pgRNA increased by 6.90-fold (t = 8.941, P = 0.0123) and 3.18-fold (t = 7.432, P = 0.0176) after 500 μg/mL of oxymatrine treatment for 2 d and 5 d, respectively.

CONCLUSION: Oxymatrine may inhibit the replication of HBV by interfering with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase.

Key words: Oxymatrine; Hepatitis B virus; Replication intermediates; Covalently closed circular DNA; Pregenomic RNA

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INTRODUCTION

Despite efficient vaccines, chronic hepatitis B virus (HBV)

infection remains a major public health problem, which involves more than 350 million people in the world[1]. Antiviral agents play a key role in the treatment of chronic hepatitis B infection by inhibiting the replication of the virus, and delaying or preventing progression to cirrhosis, hepatocellular carcinoma and death. Interferon-α (IFN-α) has been shown to be effective in suppressing HBV replication and inducing remission of liver disease[2]. Its principal antiviral mechanism has been thought to include both a direct antiviral effect and an immunomodulatory effect[3-5]. However, its efficacy is limited to a small percentage of highly selected patients and is often associated with adverse effects such as flu-like symptoms, fatigue, leucopenia, depression, anorexia, hair loss, *etc*[2]. Nucleoside analogues such as lamivudine, adefovir dipivoxil and entecavir could competitively inhibit the activity of HBV DNA polymerase or terminate the elongation of newly synthesized DNA chain by incorporation, and demonstrated potent anti-HBV efficacy *in vitro* and *in vivo*[2]. However, nucleoside analogues are also limited to certain adult patients, and related adverse effects such as renal tubular dysfunction by adefovir have also been reported[2]. In addition, viral mutation may induce drug resistance to nucleotide analogues and relapse of hepatitis B[2].

In China, many herbs or their derivatives have also been widely used in the treatment of viral hepatitis and associated complications such as liver cirrhosis and liver failure[6,7]. Oxymatrine, a type of alkaloid extracted from the herb *Sophora alopecuraides L*.[8], had shown a promising anti-HBV effect in a HBV-transfected cell line, in a HBV transgenic mice model and in patients with chronic hepatitis B[9-11]. Oxymatrine had also been found to be capable of relieving hepatic fibrosis or severe injury independently[12-14]. It has been approved for the treatment of hepatitis B by the State Food and Drug Administration of China, and is listed as one of the recommended anti-HBV agents in the Guideline for Prevention and Treatment of Chronic Hepatitis B jointly proposed by the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases[15]. Unfortunately, unlike IFN-α or antiviral nucleoside analogues, little is known about the exact mechanism or target of oxymatrine against HBV.

The replication cycle of the hepadnavirus involves a pathway by reverse transcription of an RNA intermediate[16], during which several important replicative intermediates are generated, including covalently closed circular DNA (cccDNA), pregenomic RNA (pgRNA) and progeny virus relaxed circular DNA (rcDNA), and the viral particle is secreted outside after maturation[17]. The antiviral agents usually target one or more specific sites of the HBV replication cycle. It can be reasoned that any intervention or interruption in the replication cycle of HBV will result in the fluctuation or alteration of the product occurring in the specific site, which will in turn provide clues to illuminate the target of antiviral agents. In this paper, we investigated the effect of oxymatrine on the replication cycle in the HepG2.2.15 cell line, and explored the possible antiviral target of oxymatrine.

MATERIALS AND METHODS

Compounds

Oxymatrine was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co. Ltd, China, the purity of which had been determined to be greater than 99% by high-performance liquid chromatography. The compound powder was stored at room temperature in light-resistant containers, and stock solutions were prepared when required by dissolving reagents in dimethyl sulfoxide to a concentration of 200 mg/mL and stored at -4℃.

Cell line

The HepG2.2.15 cell line, which could support persistent replication of HBV and produce intact HBV particles[18], was provided by the Molecular Viral Laboratory of Fudan University with permission from the Mount Sinai Medical Center, NY, USA. It was maintained with Dulbecco's Modified Eagle Medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 380 μg/mL G418 (Gibco-BRL, Grand Island, NY, USA) 125 μg/mL penicillin and 50 μg/mL streptomycin at 37℃ in a humidified incubator containing 50 mL/L CO_2. Then cells were harvested from the flask by treating the monolayer with 0.25% trypsin (Gibco-BRL, Grand Island, NY, USA) and 1 mmol/L EDTA, and resuspended in culture medium for further use.

Cytotoxicity

HepG2.2.15 cells were inoculated on a 96-well culture microplate (Costar, Corning Inc., NY, USA) at a density of 2×10^4/mL (200 μL for each well). After incubating for 48 h, supernatant was substituted by fresh culture medium containing serial 1:2 dilutions of oxymatrine, varying from 8000 μg/mL to 62.5 μg/mL (triplicates for each concentration), and was refreshed every other day. After treatment for 6 d, cytotoxicity of oxymatrine was determined by a MTT assay as previously described in detail[19].

Treatment of HepG2.2.15 cells with oxymatrine

HepG2.2.15 cells were inoculated in 12 flasks (75-cm^2, NUNC, Roskilde, Denmark) at a density of 2×10^5/mL (12 mL for each flask). Forty-eight hours after cell inoculation, HBV DNA could be easily detected in the culture medium. Then the culture medium was removed, and fresh culture media containing 500 μg/mL of oxymatrine were added, while the normal control group was refreshed with new culture media. At different times (2 d and 5 d) after treatment, supernatant from 3 flasks of each group were collected independently for determination of HBV surface antigen (HBsAg), HBV e antigen (HBeAg), and HBV DNA, and cells were harvested by trypsin digestion and washed 3 times with phosphate buffered solution (PBS, pH 7.3). Then cells were counted and different numbers of cells were used to determine different HBV replicative intermediates: 1×10^6 cells for cccDNA, 3×10^6 cells for

Table 1 Sequences of primers and probes for PCR or RT-PCR

Primer set			Taqman MGB probe			Target
Name	Sequence	Position[1]	Name	Sequence	Position[1]	
CCP1	5′TTCTCATCTGCCGGACCG 3′	1562-1579	TCC	5′FAM-CCTAATCATCTCTTGTTCAT-MGB 3′	1836-1855	cccDNA
CCP2	5′CACAGCTTGGAGGCTTGAAC 3′	1883-1864				
RCP1	5′TCCTCTTCATCCTGCTGCTATG 3′	404-425	TRC	5′FAM-TGTTGGTTCTTCTGGACTA-MGB 3′	437-455	rcDNA
RCP2	5′CGTGCTGGTAGTTGATGTTCCT 3′	510-489				
PGP1	5′CTCAATCTCGGGAATCTCAATGT 3′	2429-2451	TPG	5′FAM-CCTTGGACTCATAAGG-MGB 3′	2459-2474	pgRNA
PGP2	5′TGGATAAAACCTAGCAGGCATAAT 3′	2659-2636				
BAP1	5′ACGGCCAGGTCATCACCAT 3′	2396-2415	TBA	5′FAM-CAATGAGCGGTTCCG-MGB 3′	2418-2432	β-actin
BAP2	5′AGGCTGGAAGAGTGCCTCAG 3′	2438-2457				

[1]Nucleotide position in U95551 HBV sequence or M10277 β-actin gene sequence. HBV: Hepatitis B virus; cccDNA: Covalently closed circular DNA; rcDNA: Relaxed circular DNA; pgRNA: Pregenomic RNA; MGB: Minor grooving binder; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction.

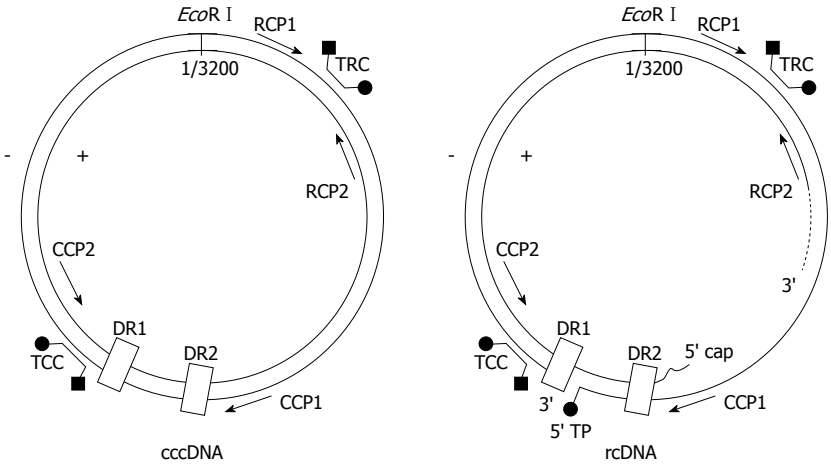

Figure 1 Schematic diagram of intracellular covalently closed circular DNA (cccDNA), relaxed circular DNA (rcDNA) and design of PCR primers and probes. When rcDNA is detected by primer set CCP1 and CCP2, new strand extension priming by CCP1 or CCP2 will cease at the site of the nick in the minus strand or gap in the plus strand, respectively, and no PCR product is generated. Therefore primer set CCP1 and CCP2 will preferentially amplify cccDNA and not (or to a lesser extent) rcDNA. Primer set RCP1 and RCP2 can amplify both cccDNA and rcDNA.

pgRNA and another 1×10^6 cells for rcDNA. At each interval, those unharvested flasks continued to be incubated with fresh culture media containing the same amount of antiviral agent as before.

Determination of HBsAg, HBeAg and HBV DNA in supernatant

Supernatant from each flask was collected at different times after oxymatrine treatment and stored at -20℃ until measurement. HBsAg and HBeAg were simultaneously detected by ELISA kits (Sino-America Biotechnology Co. Ltd, Shanghai, China) according to the manufacturer's instruction. HBV DNA in the supernatant was purified with QIAamp DNA Mini Kit (QIAGEN Inc., Chatsworth, CA, USA) following the manufacturer's instruction, and then was determined by real-time fluorescent PCR with primer RCP1/RCP2 and Taqman® minor grooving binder (MGB) probe DRC, targeting the S open reading frame (ORF) of HBV genome (Figure 1 and Table 1).

Real-time fluorescent PCR was performed on the ABI prism 7000 Sequence Detection System (Applied Biosystems Inc., USA) with following PCR cycling parameters: 2 min at 50℃ for uracil N-glycosylase incubation, 10 min at 95℃ for Hotstart Taq DNA polymerase activation, followed by 40 cycles of denaturation for 30 s at 95℃ and annealing for 60 s at 60℃. The reaction system consisted of 0.5 μL of sense primer (25 μmol/L), 0.5 μL of antisense primer (25 μmol/L), 1.25 μL of Taqman MGB probe (10 μmol/L), 25 μL of Taqman® Universal PCR master mix (2 × concentration, P/N 4304437, Applied Biosystems Incorporation, USA), 5 μL of template, supplemented with double-distilled water to a final volume 50 μL. Uniform real-time PCR system and cycling mode were utilized in the determination of cccDNA, total HBV DNA and cDNA because different prime sets and probes were designed based on the same principle for Taqman® real-time PCR technology.

Purification and quantification of intracellular cccDNA

Since HBV cccDNA is structurally similar to plasmid, intracellular cccDNA was extracted from cells by an alkali lysis procedure with Plasmid Mini Kit (QIAGEN Inc., Chatsworth, CA, USA), which would remove most of cellular chromosomal DNA and non-supercoiled rcDNA. Purified cccDNA was dissolved in 50 μL TE buffer (10 mmol/L, pH 8.0), and 10 μL product was further treated with Plasmid-Safe™ ATP-Dependent DNase (PSAD, Epicentre Technologies, Madison, WI, USA) to remove any remaining rcDNA, single-stranded virus DNA and cellular chromosomal DNA. Briefly, DNA was digested with 10 U PSAD for 1 h at 37℃ in the presence of 1 × buffer (33 mmol/L Tris-acetate pH 7.8, 66 mmol/L

potassium acetate, 10 mmol/L magnesium acetate and 0.5 mmol/L DTT) and 1 mmol/L ATP, followed by incubation in 70℃ water for 30 min to inactivate PSAD. Intracellular cccDNA was quantified by selective fluorescent PCR with primer set spanning DR1 and DR2 region, which had been documented to be capable of amplifying cccDNA more efficiently than rcDNA (10^4:1)[20,21]. Design of the primer set CCP1 and CCP2, and the Taqman® MGB probe DCC was demonstrated in Figure 1 and Table 1. The mean level of cccDNA pool of each cell at different times was calculated according to total cccDNA and the number of cells for cccDNA extraction (1×10^6).

Isolation of intracellular core particles and determination of viral DNA

Cytoplasmic core particles in HepG2.2.15 cells were isolated as described with modification[22]. Briefly, cells were incubated at 37℃ with 1 mL of lysis buffer (1 mmol/L EDTA, 0.1% Nonidet P-40, 50 mmol/L NaCl, 8% sucrose) for 10 min, then nuclei and other insoluble materials were removed by centrifugation at 15000 r/min for 2 min. Supernatants were treated with 10 U RQ1 DNase (Promega) and 0.1 μg of RNase A (Sigma) at 37℃ for 15 min, then 1/4 volume of polyethylene glycol 8000 (PEG 8000, Amresco) solution (10% PEG 8000, 0.6 mol/L NaCl) were added, followed by incubation at 4℃ for 30 min and centrifugation at 15000 r/min for 10 min. Pellets containing core particles were re-suspended in 500 μL of digestion buffer (50 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, 1% sodium dodecyl sulfate, 0.5 mg/mL proteinase K, pH 8.0) and were incubated at 50℃ for 2 h. Nucleic acids were extracted with phenol:chloroform (25:24) and then with chloroform:isoamylol (24:1), and were precipitated from the aqueous fraction with ethanol, dissolved in TE buffer (10 mmol/L Tris hydrochloride, 1 mmol/L EDTA, pH 8.5). Viral DNA in core particles was quantified by real-time fluorescent quantitative PCR with primer RCP1/RCP2 and Taqman MGB probe TRC (Figure 1 and Table 1). The mean viral DNA in core particles of each cell was calculated in the same way as cccDNA.

Extraction of total RNA and analysis of intracellular pgRNA

Total RNAs were extracted from cells with Total RNA Miniprep System (Viogene, Sunnyvale, CA, USA) and dissolved in 50 μL RNase-Free ddH_2O, and 10 μL product was treated with 8 U RQ1 DNase to eliminate DNA contamination from virus or cells. Then cDNAs were synthesized by reverse transcription with SuperScript™ Ⅲ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., San Diego, CA, USA) according to the manufacturer's instruction.

Because HBV mRNAs of different lengths (3.5, 2.4, 2.1 and 0.7 kb) are transcribed from the same cccDNA template, and ORFs overlap with each other in various regions (Figure 2), it is crucial to find a specific target region for RT-PCR in order to discriminate pgRNA from other mRNAs. In addition to its role in viral DNA replication, the pgRNA is also a bicistronic mRNA that encodes C and P proteins[22,23], which contain an approximate 1010 bp nucleotide sequence (nt1838-2850, from the end of X ORF to the origin of S ORF) not existing in the other 3 smaller mRNAs (Figure 2). The primer set PGP1/PGP2 and Taqman MGB probe TPG targeting this region was designed to detect pgRNA (Table 1 and Figure 2). Results of RNA extraction and RT-PCR were normalized with the housekeeping gene β-actin as control[24]. The β-actin cDNA of each sample was determined simultaneously by real-time PCR with primer BAP1/BAP2 and Taqman MGB probe TBA (designed on the basis of M10277 β-actin gene sequence). The mean expression level of β-actin in HepG2.2.15 was assumed to be similar to that in human tissues, namely, 5320 copies per cell[25].

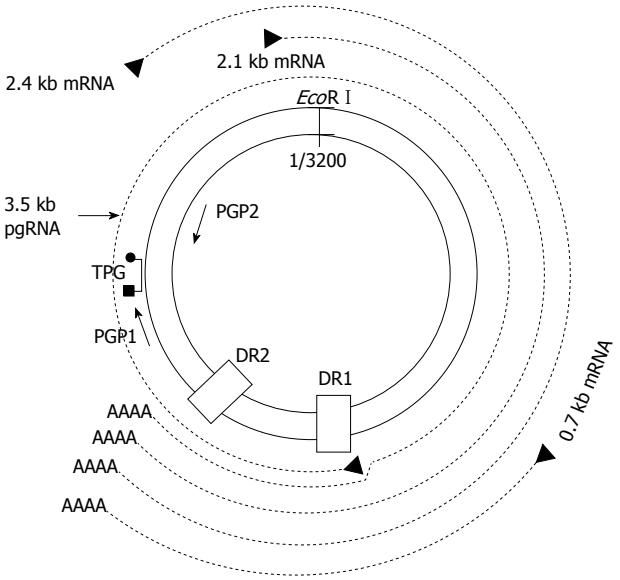

Figure 2 Schematic diagram of HBV mRNAs and design of PCR primer and probe for determining pregenomic RNA (pgRNA). Four kinds of HBV mRNAs are transcribed from the same cccDNA template and overlap with each other in various regions (dashed lines, dark triangle indicating the initiate site of transcription). The largest mRNA, 3.5 kb pgRNA, encodes both core protein and DNA polymerase of HBV. The promoter region of the DNA polymerase gene, which does not exist in the other 3 smaller mRNAs, was selected as the amplifying target for determining pgRNA by real-time RT-PCR with primer PGP1, PGP2 and Taqman MGB probe TPG.

Statistical analysis

All results were expressed as mean ± SD. Data from the treatment group and normal control group were analyzed by the Student unpaired *t* test using statistical software SPSS 10.0. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Cytotoxicity of oxymatrine to HepG2.2.15 cells

Cytotoxicity to HepG2.2.15 cells was determined with fresh culture medium containing serial 1:2 dilutions of oxymatrine. The survival percentage of HepG2.2.15 cells under different concentrations of oxymatrine is shown in Figure 3. The mean half toxic concentration (TC_{50}) of oxymatrine was 1219.66 μg/mL, calculated by the Reed-

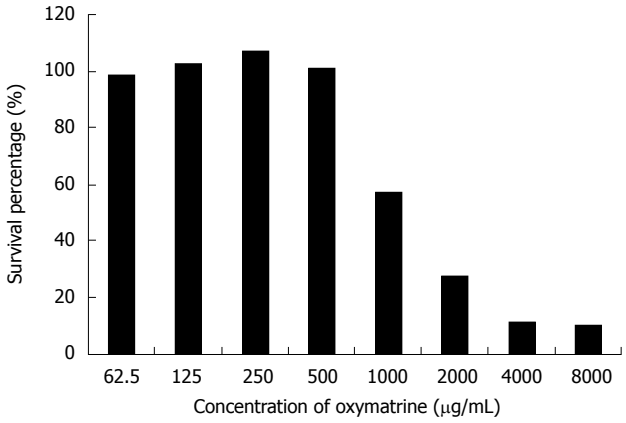

Figure 3 Survival of HepG2.2.15 cells after oxymatrine treatment. Two days after being inoculated, cells were incubated with different concentrations of oxymatrine for 6 d, and then the percent survival of cells was determined by MTT assay. The cytotoxicity experiment was repeated twice.

Muench formula[26]. In further experiments, the effect of 500 μg/mL of oxymatrine (approximately equal to the largest nontoxic concentration) on the replication cycle of HBV was analyzed *in vitro*.

Effect of oxymatrine on secretion of HBsAg and HBeAg in HepG2.2.15 cell line

As shown in Figure 4, oxymatrine could inhibit secretion of HBsAg and HBeAg by HepG2.2.15 cells. After treatment with oxymatrine for 2 d and 5 d, the mean optical density at a wavelength of 450 nm (A_{450}) was 0.096 ± 0.011 and 0.175 ± 0.016, respectively, when HBsAg in the supernatant was determined by microplate reader (Model 550, Bio-Rad), and decreased by 22.67% (t = 5.439, P = 0.0322) and 22.39% (t = 5.376, P = 0.0329) compared to the normal control (A_{450} = 0.124 ± 0.018 and A_{450} = 0.226 ± 0.022, respectively). The A_{450} of HBeAg in the supernatant after treatment for 2 d and 5 d was 0.259 ± 0.031 and 0.713 ± 0.031, respectively, which indicated that secretion of HBeAg was reduced by 55.34% (t = 9.859, P = 0.0101) and 43.97% (t = 14.080, P = 0.0050) compared to the normal control (A_{450} = 0.580 ± 0.034 and A_{450} = 1.269 ± 0.052, respectively). Of note was that the reduction in HBeAg was at least twice that of HBsAg.

Effect of oxymatrine on production of virions in the supernatant

Since each virion contains only one copy of the genome, production of hepatitis B virions in the supernatant could be measured by quantification of supernatant HBV DNA. As demonstrated in Figure 5A, treating HepG2.2.15 cells with oxymatrine resulted in a significant reduction in hepatitis B virions in the supernatant. After treatment with 500 μg/mL oxymatrine for 2 d and 5 d, the level of HBV DNA in the supernatant was (5.69 ± 0.86) × 10^4 and (7.86 ± 0.99) × 10^4 copies/mL, respectively, while in the normal control the mean level was (9.60 ± 2.98) × 10^4 and (31.85 ± 4.74) × 10^4 copies/mL, respectively. In another words, production of virions was reduced by 40.75% (t = 4.570, P = 0.0447) and 75.32 % (t = 14.460, P = 0.0047), at 2 d and 5 d, respectively.

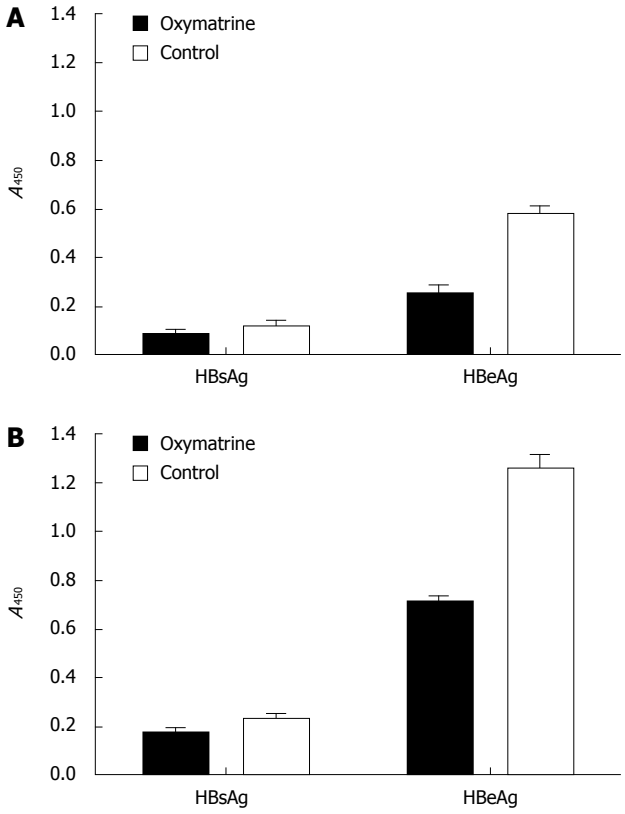

Figure 4 Effect of oxymatrine on the secretion of HBsAg and HBeAg in the HepG2.2.15 cell line. A: After treatment with oxymatrine for 2 d, HBsAg in the supernatant decreased by 22.67% compared to normal control, and HBeAg by 55.43%; B: After treatment with oxymatrine for 5 d, HBsAg in the supernatant decreased by 22.39% compared to normal control, and HBeAg by 43.97%.

Effect of oxymatrine on the formation of intracellular HBV cccDNA pool

At different times after oxymatrine treatment, the HBV cccDNA pool in HepG2.2.15 cells diminished significantly compared with the normal control group (Figure 5B). After treatment for 2 d and 5 d, the size of the intracellular HBV cccDNA pool was downregulated to 0.75 ± 0.16 and 3.50 ± 0.26 copies per cell, respectively. The decrease was 63.98% (t = 6.152, P = 0.0254) and 80.83% (t = 10.270, P = 0.0093), respectively, after incubation with oxymatrine, compared with normal controls (2.09 ± 0.23 and 18.26 ± 3.43 copies per cell, respectively).

Effect of oxymatrine on viral DNA synthesis in the intracellular core particles

The effect of oxymatrine on the intracellular production of progeny viruses was indirectly measured by determination of HBV DNA in the intracellular core particles. As shown in Figure 5C, treatment with oxymatrine could lower the level of viral DNA in core particles isolated from HepG2.2.15 cells. After oxymatrine treatment for 2 d and 5 d, viral DNA in core particles was reduced to 144.95 ± 48.78 and 302.32 ± 36.36 copies per cell, respectively,

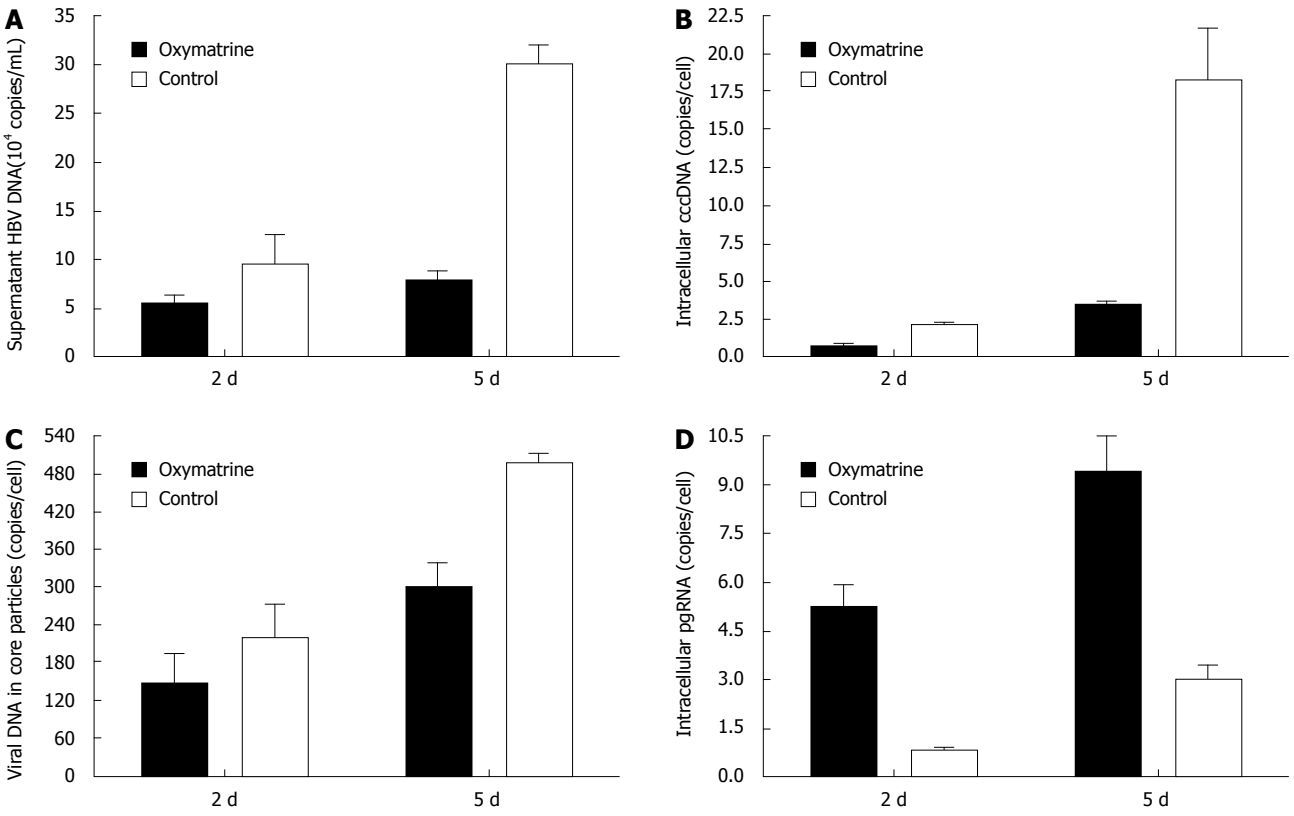

Figure 5 Effect of oxymatrine on the replication of HBV in HepG2.2.15 cell line after treatment for 2 d and 5 d. Compared to normal control, treatment with oxymatrine for 2 d and 5 d resulted in: (A) a decrease in HBV DNA in the supernatant by 40.75% and 75.32%, respectively; (B) a reduction in the intracellular HBV cccDNA pool by 63.98% and 80.83%; and (C) a reduction in viral DNA in the intracellular core particles by 34.35% and 39.24%. However, intracellular pgRNA increased by 6.90-fold and 3.18-fold (D).

which was 34.35% (t = 4.776, P = 0.0413) and 39.24% (t = 10.050, P = 0.0097) lower than that of normal controls (220.79 ± 51.73 and 497.57 ± 16.15 copies per cell, respectively). It could be noted that the reduction of viral DNA in core particles was not as great as that of the intracellular HBV cccDNA pool and HBV DNA in the supernatant after treatment with oxymatrine either for 2 or 5 d. In addition, the HBV cccDNA pool was significantly lower than that of viral DNA in core particles. After treatment with oxymatrine for 2 d and 5 d, the level of viral DNA in core particles was 193.27-fold and 86.38-fold the size of the cccDNA pool, respectively. For the normal control, it was 105.64-fold and 27.25-fold, respectively.

Effect of oxymatrine on intracellular HBV pgRNA

Unlike the other 2 replicative intermediates, the level of intracellular HBV pgRNA was upregulated after oxymatrine incubation, as shown in Figure 5D. With oxymatrine treatment for 2 d and 5 d, intracellular pgRNA accumulated to 5.25 ± 0.69 and 9.43 ± 1.13 copies per cell, respectively, which was 6.90-fold (t = 8.941, P = 0.0123) and 3.18-fold (t = 7.432, P = 0.0176) to that of normal control (0.76 ± 0.16 and 2.97 ± 0.48 copies per cell, respectively).

DISCUSSION

Because of the lack of a convenient and economic animal model with persistent HBV infection, the effect of oxymatrine on the replication cycle of HBV was investigated in the HepG2.2.15 cell line, which was established by transfecting a hepatoblastoma cell line (HepG2) with plasmids containing four 5'→3' tandem copies of the HBV genome, and could produce 42 nm Dane particles and more 22 nm spherical or filamentous particles[18]. The cell line could support the full replication cycle HBV, as evidenced by identification of replicative intermediates or products including cccDNA, HBV-specific polyadenylated RNAs (pgRNA, 2.5 kb RNA and 2.1 kb RNA), and incomplete double- and single-stranded forms of the HBV genome, none of which were necessarily dependent on the chromosomally-integrated HBV DNA[27]. In addition, HBV virions produced by the cell line were rich in endogenous polymerase activity[27] and could induce hepatitis in chimpanzees[28]. Therefore, the cell line is an appropriate model for identifying the molecular events in intracellular viral replication cycle as well as secretion of HBV particles.

As indicated by our results, secretion of HBsAg and HBeAg from HepG2.2.15 cells could be inhibited after incubation with oxymatrine for 2 d and 5 d. Of note was that secretion of HBeAg was reduced more than that of HBsAg after oxymatrine treatment either for 2 d (22.67% *vs* 55.43%) or 5 d (22.39% *vs* 43.97%). A discrepancy between the reductions in HBsAg and HBeAg has also

been observed by other researchers[9,29]. HBsAg is encoded by S ORF of the viral genome, and is organized into spherical or filamentous HBsAg particles outside infected cells without a viral genome, which typically outnumber virions by 1000:1 to 10 000:1[30]. Thus, expression of HBsAg can be independent of the replication of HBV. HBeAg is translated from the same template as core protein and polymerase, pgRNA, which is also the template for synthesis of progeny virus. In contrast to HBsAg, although HBeAg plays no role in viral assembly and its function is not clear[30], expression and secretion of HBeAg were found to be associated with active replication of HBV[31]. Therefore, inhibition of HBV replication would inevitably reduce the secretion of HBeAg while it may not affect or have a smaller effect on the secretion of HBsAg, as evident by the difference between the reductions in HBsAg and HBeAg by oxymatrine.

Consistent with previous research *in vitro*[9,19,29], definite inhibition by oxymatrine of the replication of HBV was observed in our investigation. As far as we know, this was the first time that the effect of oxymatrine on production of virions in the supernatant and intracellular synthesis of the viral genome was simultaneously investigated. After treatment with oxymatrine for 2 d and 5 d, production of virions in the supernatant was reduced by 40.75% and 75.32%, while viral DNA synthesis in intracellular core particles was 34.35% and 39.24% lower than that of normal control. It appeared that extracellular viral DNA was more likely to be reduced by oxymatrine than intracellular viral synthesis. A similar effect has also been observed in cell lines treated with lamivudine or clevudine[32], and in patients with chronic hepatitis B who were under antiviral therapy with adefovir or entecavir[33,34]. A possible explanation for this may be that competitive inhibition of activity of HBV DNA polymerase or premature chain termination by these antiviral agents may result in predominant immature viral particles containing only intact or non-intact single-stranded viral DNA, which could not be discriminated from mature virions containing relaxed circular double-stranded DNA by routine PCR or fluorescent PCR. Therefore, the reduction in mature virions may not be intrinsically reflected in the determination of viral DNA in core particles by PCR. In contrast, because only mature virions could be secreted outside infected cells[16], reduced intracellular production of mature virions could be more sensitively identified by detection of supernatant HBV DNA in cell lines or serum HBV DNA *in vivo*.

The effect of oxymatrine on intracellular HBV cccDNA was also first reported in this research. HBV cccDNA is the first replicative intermediate generated after HBV entering into hepatocytes, which indicates the origination of intracellular HBV replication and successful establishment of HBV infection[17]. Reductions varying from 0.8 $\log_{10}$ to 2.8 $\log_{10}$ in the intrahepatic cccDNA have been reported in patients receiving mono- or combined antiviral therapy with IFN-α or nucleos(t)ide analogues for 48 wk[33-35], and persistence of cccDNA in the nuclei of hepatocytes after withdrawal of antiviral agents was believed to be primarily responsible for the reoccurrence of hepatitis B[36]. Therefore, the effect on nuclear cccDNA was an indispensable index when evaluating an anti-HBV agent, and a predictor of a sustained antiviral response to therapy[37]. In our research, a marked decline in the intracellular cccDNA pool was observed after oxymatrine treatment for 2 d and 5 d (63.98% and 80.83%, respectively). The conversion of viral genome into cccDNA did not depend on viral polymerase activity[20], and formation of the nuclear cccDNA pool in the nuclei of hepatocytes had been shown to be mainly dependent on recycling of cytoplasmic mature progeny virions into nuclei of hepatocytes[38,39]. Therefore, the reduction in the cccDNA pool induced by oxymatrine may be attributed to reduction in cytoplasmic mature virions rather than direct inhibition of the conversion of rcDNA into cccDNA. It should be noted that, in contrast to hepatocytes in the liver, HepG2.2.15 cells are actively dividing, and whether cccDNA can survive cell division or not is still unknown[40]. The reduction in the cccDNA pool should not be attributed to cell proliferation, because the survival of HepG2.2.15 cells incubated with 500 μg/mL oxymatrine was found to be approximately 100% in the cytotoxicity experiment (Figure 2), and the cell proliferation rate after oxymatrine treatment was not significantly different to that of normal control when assessed by the total number of cells acquired from flasks at each time (data not shown).

Interestingly, although significantly lower than that of the normal control group ($P < 0.05$), the cccDNA pool after oxymatrine continued to expand slowly (from 0.75 copies per cell to 3.50 copies per cell). This was probably because production of virions in the cytoplasm was not completely inhibited, and more cytoplasmic rcDNAs were recycled into the nucleus instead of being secreted out so as to compensate for the loss of the cccDNA pool and maintain intracellular infection. The "wisdom" of HBV to maintain an intracellular existence by conserving cccDNA could be corroborated by other facts. It has been reported that even in chimpanzees or patients with sustained clearance of serum HBsAg and negative serum HBV DNA, residual cccDNA could still be detected in their hepatocytes[41,42].

In our research, a special strategy was taken to determine intracellular cccDNA, which integrated extraction by alkali lysis, purification with Plasmid-Safe™ ATP-dependent DNase (PSAD) and quantitative real-time PCR with selective primer set. Because HBV cccDNA is similar to plasmid in spatial structure and physicochemical characteristics, a procedure for purification of plasmid DNA with a kit based on alkali lysis was adopted, which would irreversibly denature double-stranded DNA species (including HBV DNA integrated into the cellular chromosome and viral rcDNA) that were not covalently closed[43,44]. PSAD can selectively hydrolyze linear double-stranded (ds) DNA to deoxynucleotides at slightly alkaline pH, and with a lower efficiency, hydrolyzes linear and closed circular single-stranded DNA. We had observed that overnight incubation of total chromosomal DNA from 20 mg of

liver tissues with 10 U PSAD could digest it to an invisible level in agarose electrophoresis, and approximately 1 $\log_{10}$ reduction was observed by real-time PCR when HBV genome with 10^8 to 10^6 hepatitis B virions was treated with 10 U PSAD for 1 h (data not shown). PSAD was also frequently used by other researchers[21,32,33,35] to minimize the background rcDNA before detection of cccDNA with selective PCR, which would preferentially amplify cccDNA rather than rcDNA (10^4:1)[20,21].

After oxymatrine incubation for 2 d and 5 d, intracellular pgRNA increased by 6.90-fold and 3.18-fold, respectively. It seemed a little incomprehensible why pgRNA accumulated distinctively in spite of a decline in the cccDNA pool and reduction of DNA synthesis in core particles. Two possible targets in the intracellular replication cycle of HBV interference by oxymatrine might account for such result. First, the packaging process of pgRNA into the protein nucleocapsid was interrupted by oxymatrine, and pgRNA accumulated in the nucleus or cytoplasm of hepatocytes, which resulted in further reduction of DNA synthesis in core particles and then nuclear cccDNA. Second, pgRNA was packaged into nucleocapsid as normal, but the activity of virus DNA polymerase was suppressed by oxymatrine. It is well known that accompanying extension of viral minus-strand DNA by reverse transcription with pgRNA as template (catalyzed by reverse transcriptase activity of HBV polymerase), pgRNA is degraded synchronously from pgRNA/minus-strand DNA hybrids by the RNase H activity of viral polymerase[17,45]. Therefore, suppression of viral DNA polymerase would lead to reduction of rcDNA and cccDNA and less pgRNA degradation compared to the normal control group. This hypothesis could be evidenced by the phenomena discovered by other investigators, who reported that duck HBV reverse transcriptase expressed by a recombinant P vector would suppress viral pregenomic RNA accumulation by 3-4-fold in an LMH cell line[24]. Whether the packaging process of pgRNA or viral DNA polymerase was the exact antiviral target of oxymatrine remains to be further investigated.

Very few investigations had studied the effect of antiviral agents on intracellular HBV RNAs as far as we know. In HBV-transgenic mice, animals treated with adefovir resulted in a significant reduction in serum and intrahepatic HBV DNA, but mean relative intrahepatic HBV RNA ($\log_{10}$ pg/μg ± SD), seemed to be a little higher than that of saline-control animals (5.8 ± 2.5 *vs* 4.7 ± 2.4) after treatment, although the difference was not statistically significant[46]. In the same research, animals treated with lamivudine had a significant reduction in serum HBV DNA, and slight but insignificant reduction in intracellular HBV DNA, and HBV RNA seemed to be even higher than that of saline-control animals (7.0 ± 1.4 *vs* 4.7 ± 2.4) although the difference was also statistically significant. It should be noted that the author did not clarify which fragment of HBV DNA had been chosen as a probe for detecting HBV RNA by Northern blot, so it was hard to know which of the 4 HBV mRNAs (3.5 kb, 2.4 kb, 2.1 kb and 0.07 kb) may contribute to the alteration of HBV RNA in the result. Other nucleoside analogues, such as 5-fluoro-2',3'-dideoxy-3'-thiacytidine (5-FSddC), failed to affect intrahepatic HBV-specific RNAs (3.5 kb, 2.5 kb and 2.1 kb) when detected by Northern blot, although they reduced intracellular viral DNAs (rcDNA and single-stranded DNA) in a dose-dependent manner[47].

In conclusion, our research demonstrated an inhibitory effect of oxymatrine on the replication cycle of HBV, which may be attributed to interference with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase. The independent effect of oxymatrine on these 2 targets remains to be studied further. In addition, comparative analysis of the chemical or spatial structure between oxymatrine and nucleoside analogues may help illuminate the antiviral mechanism of oxymatrine.

COMMENTS

Background

It is the consensus that anti-hepatitis B virus (HBV) therapy plays a decisive role in slowing disease progress, prolonging the survival, and improving the prognosis of patients with chronic hepatitis B. Anti-HBV agents now available include interferon-α and several nucleos(t)ide analogues. Although many patients have benefited from them, the adverse effect of interferon-α, virus-resistance to nucleos(t)ide analogues as well as the high cost of these agents have limited their utilization. Therefore, it is very necessary to screen for more cost-effective antiviral agents.

Research frontiers

China has unique resources for screening for candidate anti-HBV drugs in traditional medicine. In this area, most researchers work hard to find new derivatives or mono-components from various Chinese herbs to test their anti-HBV effect *in vitro* or *in vivo*. However, not enough importance has been attached to identification of the antiviral mechanism of the agents that seem to have promising anti-HBV activity.

Innovations and breakthroughs

Oxymatrine is a type of alkaloid extracted from the herb *Sophora alopecuraides L.* Prior research concerning oxymatrine involved determining its anti-HBV effect in cell lines, in animals and in double-blind, randomized, multicenter clinical trials. Some researchers had observed its effect in relieving hepatic fibrosis or severe liver injury. Unfortunately, no research had tried to explore the exact antiviral target of oxymatrine, and little was known about the mechanism of oxymatrine against HBV until now, which is a major obstacle to its marketing. In this paper, the authors reported the effect of oxymatrine on the main steps of the HBV replication cycle. The change in different replicative intermediates after oxymatrine incubation was documented and analyzed for the first time. Based on the results, the authors proposed that oxymatrine may inhibit the replication of HBV by interfering with the process of packaging pregenomic RNA (pgRNA) into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase.

Applications

Although the authors have not confirmed the exact target of oxymatrine in inhibiting the replication of HBV in this article, the preliminary results proposed 2 candidate targets for further research.

Peer review

In this paper, the effect of oxymatrine on the replication cycle in the HepG2.2.15 cell line was investigated, and the possible antiviral targets of oxymatrine were explored. The inhibitory effect of oxymatrine on the replication cycle of HBV, interfering with the process of packaging pgRNA into the nucleocapsid, or inhibiting the activity of the viral DNA polymerase were demonstrated.

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