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Anti-HBV effect of liposome-encapsulated matrine *in vitro* and *in vivo*

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Supported by the Natural Science Foundation of Guangdong Province, No.B990353

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Received: 2004-01-09 **Accepted:** 2004-02-21

Abstract

AIM: To study the anti-HBV effect of liposome-encapsulated matrine (Lip-M) *in vitro* and *in vivo*.

METHODS: 2.2.15 cell line was cultured *in vitro* to observe the effect of Lip-M and matrine on the secretion of HBsAg and HBeAg. The toxicity of Lip-M and matrine to 2.2.15 cell line was also studied by MTT method. In *in vivo* study, drug treatment experiment was carried out on the 13th day after ducks were infected with duck hepatitis B virus (DHBV). The ducks were randomly divided into 4 groups with 5-6 ducks in each group. Lip-M and matrine were given to DHBV-infected ducks respectively by gastric perfusion. Four groups were observed: group of Lip-M (20 mg/kg), group of Lip-M (10 mg/kg), group of matrine (20 mg/kg) and group of blank model. The drug was given once daily for 20 d continuously, and normal saline was used as control. The blood was drawn from the posterior tibial vein of all ducks before treatment (T_0), after the medication for 5 (T_5), 10 (T_{10}), 15 (T_{15}), 20 (T_{20}) d and withdrawal of the drug for 3 d (P_3). The serum samples were separated and stored at -70°C , DHBV-DNA was detected by the dot-blot hybridization.

RESULTS: After addition of Lip-M and matrine to 2.2.15 cell line for eleven d, the median toxic concentration (TC_{50}) of Lip-M and matrine was 7.29 mg/mL and 1.33 mg/mL respectively. The median concentration (IC_{50}) of Lip-M to inhibit HBsAg and HBeAg expression was 0.078 mg/mL and 3.35 mg/mL respectively. The treatment index (TI) value of Lip-M for HBsAg and HBeAg was 93.46 and 2.17 respectively, better than that of matrine. The DHBV-infected duck model treatment test showed that the duck serum DHBV-DNA levels were markedly reduced in the group of Lip-M (20 mg/kg) after treated by gastric perfusion for 10, 15 and 20 d (0.43 ± 0.22 vs 0.95 ± 0.18 , $t = 4.70$, $P = 0.001 < 0.01$, 0.40 ± 0.12 vs 0.95 ± 0.18 , $t = 6.34$, $P = 0.000 < 0.01$, 0.22 ± 0.10 vs 0.95 ± 0.18 , $t = 8.30$, $P = 0.000 < 0.01$), compared to the group of matrine (20 mg/kg) (0.43 ± 0.22 vs 0.79 ± 0.19 , $t = 3.17$, $P = 0.01 < 0.05$, 0.40 ± 0.12 vs 0.73 ± 0.24 , $t = 3.21$, $P = 0.009 < 0.05$, 0.22 ± 0.10

vs 0.55 ± 0.32 , $t = 2.27$, $P = 0.046 < 0.05$), and the control (0.43 ± 0.22 vs 0.98 ± 0.29 , $t = 3.68$, $P = 0.005 < 0.01$, 0.40 ± 0.12 vs 0.97 ± 0.30 , $t = 4.26$, $P = 0.002 < 0.01$, 0.22 ± 0.10 vs 0.95 ± 0.27 , $t = 5.76$, $P = 0.000 < 0.01$). After the treatment for 20 d and withdrawal of the drug for 3 d, duck serum DHBV-DNA level in the group of Lip-M (10 mg/kg) markedly reduced (0.56 ± 0.26 vs 0.95 ± 0.38 , $t = 5.26$, $P = 0.003 < 0.05$, 0.55 ± 0.25 vs 0.95 ± 0.38 , $t = 5.52$, $P = 0.003 < 0.05$), and the difference was significant as compared with the control (0.56 ± 0.26 vs 0.95 ± 0.27 , $t = 2.37$, $P = 0.042 < 0.05$, 0.55 ± 0.25 vs 0.89 ± 0.18 , $t = 2.55$, $P = 0.031 < 0.05$), but not significant as compared with the group of matrine (20 mg/kg). After withdrawal of the drug for 3 d, the levels of DHBV-DNA did not relapse in both groups of Lip-M.

CONCLUSION: Lip-M can evidently inhibit the replication of hepatitis B virus *in vitro* and *in vivo*; its anti-HBV effect is better than that of matrine.

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Key words: Duck hepatitis B virus; Matrine; Liposome; Virus Replications

Li CQ, Zhu YT, Zhang FX, Fu LC, Li XH, Cheng Y, Li XY. Anti-HBV effect of liposome-encapsulated matrine *in vitro* and *in vivo*. *World J Gastroenterol* 2004; 11(3): 426-428

<http://www.wjgnet.com/1007-9327/11/426.asp>

INTRODUCTION

Matrine, as one of the ingredients of traditional herb-Sophora flavescens, has been shown to have good anti-HBV^[1,2], anti-inflammation^[3,4], anti-hepatocyte injury^[5] and anti-liver fibrosis^[6] effects, and is widely used for treating viral hepatitis B and liver fibrosis in China. To enhance the anti-HBV effect of matrine, we manufactured Lip-M, and used 2.2.15 cell line and DHBV-infected duck models to observe and evaluate its anti-HBV activity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Drugs

Lip-M was prepared by Pharmaceutical Research Section of School of Chinese Materia Medica, Guangzhou University of Traditional Chinese Medicine, each injection contained 10 mg/mL matrine. Matrine was provided by Guangzhou Mingxing Medical Factory (Batch number: MB3001). It was a transparent liquid injection, and each injection contained 50 mg/5mL matrine.

2.2.15 cell line

2.2.15 cells were HBV-DNA transduced human liver cancer cells, which were provided by Viral Research Section of Guangzhou Air Force Hospital. The cells were incubated in DMEM supplemented with 100 mL/L fetal bovine serum, 100 u/mL

Table 1 Inhibitory effect of Lip-M on secretion of HBsAg, HBeAg in 2.2.15 cell line

Drug	TC ₅₀ (mg/mL)	HBsAg		HBeAg	
		IC ₅₀ (mg/mL)	TI	IC ₅₀ (mg/mL)	TI
Lip-M	7.29	0.078	93.46	3.35	2.17
Matrine	1.33	<0.078	>17.05	>10	<0.13
Liposome	>10	>10	1	>10	1

Table 2 Inhibitory effect of Lip-M on DHBV-DNA level *in vivo* (mean±SD)

Group	Duck <i>n</i>	A value					
		T ₀	T ₅	T ₁₀	T ₁₅	T ₂₀	P ₃
Control	5	1.07±0.19	1.39±0.33	0.98±0.29	0.97±0.30	0.95±0.27	0.89±0.18
Lip-M 20 mg/kg	6	0.95±0.18	1.08±0.21	0.43±0.22 ^{b,d,e}	0.40±0.12 ^{b,d,e}	0.22±0.10 ^{b,d,e}	0.20±0.10 ^{b,d,e}
Lip-M 10 mg/kg	6	0.95±0.38	1.07±0.68	0.75±0.33	0.68±0.34	0.56±0.26 ^{a,c}	0.55±0.25 ^{a,c}
Matrine 20 mg/kg	6	1.08±0.38	1.22±0.40	0.79±0.19	0.73±0.24	0.55±0.32 ^{a,c}	0.59±0.35 ^{a,c}

^a*P*<0.05, ^b*P*<0.01 *vs* the control; ^c*P*<0.05, ^d*P*<0.01 *vs* level of the group at different times (T₁₀, T₁₅, T₂₀, P₃) after and before treatment (T₀); ^e*P*<0.05 *vs* group of matrine (20 mg/Kg).

penicillin, 100 u/mL streptomycin, 380 ug/mL G418 and 0.03% L-glutamine, and cultured at 37 °C in an incubator containing 50mL/L CO₂.

Treatment of cells

After digested by 0.06% trypsin, 3×10⁴/0.1 mL of 2.2.15 cells were incubated in a 96-well microplate for 2 d, the supernatant was substituted by culture liquid containing drugs of different concentrations, each concentration in 4 wells, 8 concentrations (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 mg/mL) of Lip-M and matrine diluted by culture liquid and matrine were used as controls. After 11d, the concentrations of HBsAg and HBeAg were determined in the supernatant. Meanwhile, the survival rates of cells were determined by MTT method. Four wells contained culture liquid as the empty controls, 4 wells contained 2.2.15 cells as the cell controls.

Detection of HBsAg and HBeAg

HBsAg and HBeAg in the culture medium were determined according to the protocols by ELISA kits (Hua mei Biological Technical Co.Ltd.).

Determination of survival rates of cells

Survival rates of cells were detected by MTT method^[7]. The supernatant was aspirated and MTT was added to the 2.2.15 cell medium, and then cultured for 4 h, DMSO was added till it was dissolved completely, then absorbance (A) of the lysate was read at 595 nm.

Calculation of values of TC₅₀

Toxicity cell percentage (TCP%) = [(A value of cell control - A value of drug group) / (A value of cell control - A value of empty control)] × 100%.

TC₅₀ = antilog[B + (50 - <50% TCP) × C / (>50% TCP - <50% TCP)]^[7], where A = log>50% drug toxicity concentration, B = log<50% drug toxicity concentration, C = A - B.

Calculation of values of IC₅₀

Antigen inhibition percentage (AIP%) = [(A value of cell control - A value of drug group) / (A value of cell control - A value of empty control)] × 100%.

IC₅₀ = antilog[B + (50 - <50% AIP) × C / (>50% AIP - <50% AIP)]^[8], where A = log>50% drug concentration, B = log<50% drug concentration, C = A - B.

Calculation of values of TI

TI = TC₅₀/IC₅₀, which was used for evaluating the clinical application prospect of the drug^[8]: TI<1, toxic, ineffective; TI = 1-2, effective, with some toxicity; TI>2, better effect, with low toxicity. The higher the TI, the better the effect.

Animals

Young ducks (males and females were not distinguished) were bought from Guangzhou Tangxi Chicken Farm.

DHBV infection and drug treatment experiment

Each duck, aged 1 d, was injected into its abdominal cavity with 0.2 mL of serum from Shanghai ducks with positive DHBV-DNA serology. After 7 d, their blood was drawn, serum samples were separated and stored at -70 °C.

Drug treatment experiment was carried out on the 13th d after ducks were infected with DHBV. The ducks were randomly divided into 4 groups with 5-6 ducks in each group. Lip-M and matrine were given to DHBV-infected ducks, respectively, by gastric perfusion. Four groups were observed: group of Lip-M (20 mg/kg), group of Lip-M (10 mg/kg), group of matrine (20 mg/kg) and group of blank model. The drug was given once daily for 20 d continuously, and normal saline was used as control. The blood was drawn from the posterior tibial vein of all ducks before treatment (T₀), after the medication for 5 (T₅), 10 (T₁₀), 15 (T₁₅), and 20 (T₂₀) d and withdrawal of the drug for 3 d (P₃). The serum samples were separated and stored at -70 °C.

Detection of DHBV-DNA

The duck serum DHBV-DNA level was tested by the dot-blot hybridization method^[9], optical absorbance (A) was read at 590 nm, and the value of the dot was the DHBV-DNA level of the sample.

RESULTS

Effect of Lip-M on secretion of HBsAg and HBeAg in 2.2.15 cell line *in vitro*

As shown in Table 1, Lip-M had a remarkable inhibitory effect on secretion of HBsAg and HBeAg in 2.2.15 cell line, their values of treatment index (TI) were 93.46 and 2.17 respectively indicating that Lip-M was effective and low toxic.

Our results showed that Lip-M had a better inhibitory effect on secretion of HBeAg in 2.2.15 cell line, and its TI was greater than 2, which was significantly higher than that of matrine.

Inhibitory effect of Lip-M on DHBV replication in vivo

As shown in Table 2, the duck serum DHBV-DNA levels markedly reduced in the group of Lip-M (20 mg/kg) after treating for 10, 15 and 20 d ($P < 0.01$), compared to the group of matrine (20 mg/kg) ($P < 0.05$), and the control ($P < 0.01$). After treatment for 20 d and withdrawal of the drug for 3 d, the levels of duck serum DHBV-DNA in the group of Lip-M (10 mg/kg) markedly reduced ($P < 0.05$) as compared with the control ($P < 0.05$), but the difference was not significant as compared with the group of matrine (20 mg/kg). After withdrawal of the drug for 3 d, the levels of DHBV-DNA did not relapse in both groups of Lip-M.

DISCUSSION

Now, drugs that have very good anti-HBV effects are to be used in clinical treatment; therefore, it is necessary to look for the new valid medicines and treatment methods. Target therapy has good curative effects by combining drugs with carriers which deliver the drugs to the particular organ and cells^[10]. Liposome, as a pharmacological carrier, is mainly absorbed in the reticuloendothelial system, especially in liver and spleen. It can reduce the dosage of medicine, strengthen its therapeutic effect and alleviate its side effects. Moreover, all kinds of natural phospholipids have the functions of protecting liver cells and strengthening the cellular immunity. Therefore, after encapsulated by liposome, the anti-virus medicine has three major curative effects: target anti-viruses, immunity regulation and liver cell protection^[11-13].

At present, 2.2.15 cell line and DHBV-infected ducks are still the two main models used for evaluating the anti-HBV effect of drugs^[9,14]. Results of animal experiment study showed that matrine had a good anti-DHBV activity^[15]. Interfering with the synthesis of HBV-DNA and inducing the production of IFN- α have been regarded as the main anti-HBV mechanism of matrine^[16,17]. Our results showed that Lip-M could alleviate the toxicity of matrine to 2.2.15 cell line, its TC_{50} was obviously higher than that of matrine, and its inhibitory effect on HBeAg secretion was also better than that of matrine. Therefore, treatment index values of Lip-M for HBeAg were higher than those of matrine, indicating that as a carrier, liposome can alleviate the toxicity and strengthen the treatment effect of matrine *in vitro*.

The results of our study showed that matrine was effective in suppressing DHBV replication of DHBV-infected duck models by oral perfusion. The duck plasma DHBV-DNA levels markedly decreased in the high dosage group of Lip-M (20 mg/kg) after treating for 10, 15, 20 d, and withdrawal of the drug for 3 d compared to the same dosage (20 mg/kg) group of matrine. After treatment for 20 d and withdrawal of the drug for 3 d, the duck plasma DHBV-DNA levels in low dosage group of Lip-M (10 mg/kg) had no significant difference as compared with the matrine group (20 mg/kg), and the levels of serum DHBV-DNA did not relapse in the two groups of Lip-M after withdrawal of the drug for 3 d. It is suggested that liposome, a

pharmacological carrier, can decrease the dosage of matrine and enhance its anti-DHBV effects.

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Edited by Wang XL and Kumar M