PHYTOTHERAPY RESEARCH
Phytother. Res. **24**: 1627–1630 (2010)
Published online 7 June 2010 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.3155

In Vitro Antiviral Activity of Lutein against Hepatitis B Virus

Ran Pang,[1] Jun-Yan Tao,[2] Shu-Ling Zhang,[1]* Lei Zhao,[1] Xin Yue,[2] Yue-Feng Wang,[1] Pian Ye,[1] Ji-Hua Dong,[3] Ying Zhu[2] and Jian-Guo Wu[2]

[1]Department of Hepatology and Infectious Disease, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, P.R. China
[2]State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, 430072, P.R. China
[3]Central Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, P.R. China

Despite the availability of an effective vaccine, the hepatitis B virus (HBV) infection and its treatment remains one of the foremost public health problems in the world. The present study was performed in order to investigate the anti-HBV activity of lutein *in vitro.* The antiviral activity of lutein was examined by detecting the levels of HBsAg, HBeAg and extracellular HBV DNA in stable HBV-producing human hepatoblastoma HepG2 2.2.15 cells. It was found that lutein effectively suppressed the secretion of HBsAg from HepG2 2.2.15 cells in a dose-dependent manner, and it also suppressed the amount of extracellular HBV DNA. A luciferase reporter gene assay was used to determine the effects of lutein on the activities of HBV promoters. The results showed that lutein inhibited the activity of HBV full-length promoter (Fp). These data indicate that lutein possesses an anti-HBV activity and exerts its antivirus effects via inhibition of HBV transcription. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: hepatitis B virus; lutein; HBsAg; HBeAg; HBV DNA; HBV promoter.

INTRODUCTION

The hepatitis B virus (HBV), a member of the Hepadnaviridae family, contains a partial double-stranded circular DNA genome of 3.2 kb. Its genome has a compact organization, with four overlapping reading frames running in one direction and no noncoding regions. This unconventional genome structure reflects the unconventional mode of its replication which involves reverse transcription of an RNA pregenome of 3.5 kb as a first step (Arbuthnot *et al.*, 2005). The transcription of HBV genome is controlled by core, s1, s2 and X gene promoters. These promoters are regulated by enhancer 1 and enhancer 2 (Choi *et al.*, 1999).

Long-term HBV infection can result in cirrhosis and hepatocellular carcinoma (HCC), either of which can lead to a liver-related death (Park *et al.*, 2006). Although several pharmacological therapies are being implemented to treat hepatitis B currently, effective antiviral therapy against HBV infection has not been fully developed yet. Moreover, their serious side effects are still unsatisfactory (Liaw, 2002). In light of these facts, it is important and urgent to search for novel effective antiviral agents.

Lutein, a major dietary carotenoid, is abundant in green leafy vegetables, orange colored fruits, and egg yolks (Stringham and Hammond, 2005). Like other carotenoids, lutein also possesses several important biological activities. Acting as a powerful antioxidant, lutein plays an important role in ocular health as well as the prevention of cardiovascular disease, stroke and lung cancer with low toxicity (Harikumar *et al.*, 2008). Lutein is also reported to possess anticarcinogenic activity, antiinflammatory activity (Kozuki *et al.*, 2000; Narisawa *et al.*, 1996; Ukiya *et al.*, 2006). However, the antiviral activity of lutein remains uninvestigated. In this study, it was demonstrated that lutein could exert anti-HBV activity by decreasing the HBsAg expression and viral DNA replications with little cytotoxicity *in vitro*, and the antiviral activity of lutein is associated with the HBV promoter activities.

* Correspondence to: Shu-Ling Zhang, Department of Hepatology and Infectious Disease, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, P.R. China.
E-mail: tj8285@163.com

MATERIALS AND METHODS

Lutein and 3TC. The lutein used in this study was supplied by Wuhan Jianmin Pharmaceutical Group Co., Ltd, China. Before beginning the experiment, the lutein was dissolved in dimethylsulfoxide (DMSO) and then diluted with culture medium to the desired working concentration. Lamivudine (3TC), obtained from GlaxoSmithKline (Research Triangle Park, NC), was used as the positive control.

Cell cultures. HepG2 and HepG2 2.2.15 were procured from China Center for Typical Culture Collection (CCTCC) (Wuhan, China). The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin (Gibco, Grand Island, NY). The cells

Received 19 August 2009
Revised 29 January 2010
Accepted 02 February 2010

were subcultured every 3 days by detaching the cells with pancreatin (0.5 mg/mL) followed by a change of medium on the following day.

Cytotoxicity assays. The cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well and cultured at 37°C for 24 h. Then the culture medium was removed and replaced with fresh medium supplemented with various concentrations of lutein every other day. After 9 days of culture, the cytotoxic effect of lutein was evaluated by MTT assay. Four hours prior to termination of the cultures, 20 μL MTT (5 mg/mL in a phosphate-buffered saline, pH 7.4) was added to the monolayer of cells. After incubation at 37°C for 4 h, 150 μL DMSO was added to each well to solubilize the formazan. The optical density (OD) at 490 nm was measured using an automatic plate reader (BIOTek, Elx800).

Determination of HBsAg and HBeAg. The HepG2 2.2.15 cells were plated at a density of 1×10^4 cells per well on 96-well cell culture plates and were routinely cultured. Different concentrations of lutein were supplemented to the medium in triplicate 2 days after the cells were plated. After incubation with lutein for 3, 5, 7 and 9 days, the supernatants were collected. The samples were centrifuged at 5000 rpm for 10 min to drop cellular debris, and then immediately used for HBsAg or HBeAg assays. The concentrations of HBsAg and HBeAg were quantified by a commercial ELISA kit (Kehua Bio-engineering Corporation, Shanghai, China) according to the manufacturer's protocol. Data were calculated as the percentage of control by the formula: (% of control) = (ODT)/(ODC) × 100%, where ODT and ODC indicated the cell number adjusted OD of the test drugs and the control, respectively.

Determination of HBV DNA. The quantity of extracellular HBV DNA in the supernatant was detected by real-time PCR (ABI PRISM 7300 Sequence Detector, PE Biosystems) based on the TaqMan technology. Viral DNA was extracted from culture supernatant and the amount of hepatitis B viral DNA was quantified using a diagnostic kit (DaAn Gene Co. Ltd, Guangzhou, China) according to the manufacturer's protocol. A series dilution of known amounts of HBV–DNA was used as a control. The cycling program was: 93°C for 2 min, 10 cycles of 93°C for 45 s and 55°C for 60 s, 30 cycles of 93°C for 30 s and 55°C for 45 s.

Plasmid constructions and HBV promoter luciferase reporter assay. There are five HBV promoters concerned in our study – Core promoter (Cp) [nucleotides (nt) 1603–1819 on GenBank accession no. U95551], S1 promoter (S1p) (nt 2700–2830), S2 promoter (S2p) (nt 2950–3174), X promoter (Xp) (nt 935–1361) and full-length promoter (Fp) (nt 123–1875) (Choi *et al.*, 1999; Moolla *et al.*, 2002). The promoters were amplified by PCR from HepG2.2.15 cell genomes that contain HBV genome (U95551, ayw subtype). To generate pCp-Luc, pS1p-Luc, pS2p-Luc, pXp-Luc and pFp-Luc, the promoter regions of HBV were cloned upstream of the luciferase reporter gene of pGL3-basic (Promega, USA), respectively. The HepG2 cells were transiently transfected with the reporter vector using Sofast transfection reagent (Xiamen Sunma Biotechnology, China). After 8 h of transfection, the cells were treated with 40 μg/mL lutein for 48 h. The transfected cells were collected and lysed in order to perform a luciferase activity assay. HBV promoter activities were determined by measuring luciferase activity in a TD-20/20 luminometer (Turner BioSystems, USA) using the Luciferase Reporter Assay System (Promega, USA).

Statistical analysis. Statistical analysis was performed using the SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between the test samples and control. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity of lutein

The cytotoxicity of lutein on the cell viability of HepG2 2.2.15 cells was evaluated by using the MTT assay. As shown in Fig. 1, there was no significant difference in the cell viability between lutein-treated groups whose concentrations were below 40 μg/mL and the control group. But higher concentrations of lutein were demonstrated to be cytotoxic.

Anti-HBV activity of lutein *in vitro*

The HBsAg and HBeAg in the supernatant were determined by ELISA assay. The results indicated that lutein could inhibit the secretion of HBsAg from HepG2 2.2.15 cells ($p < 0.05$; Fig. 2), and had little effect on the HBeAg secretion (Fig. 3). To further confirm the antiviral activity of lutein in HepG2 2.2.15 cells, the extracellular HBV DNA levels were evaluated by real-time PCR. Consistent with the inhibitory effects on HBsAg secretion, lutein treatment of HepG2 2.2.15 cells resulted in a reduction of the extracellular HBV DNA levels ($p < 0.05$; Fig. 4).

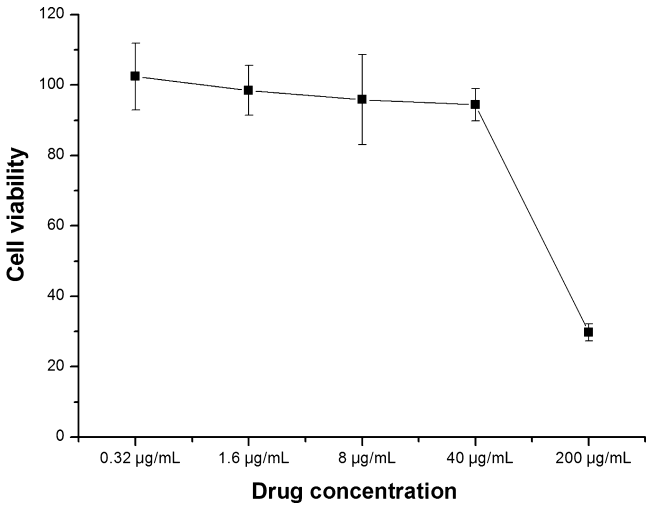

Figure 1. The cytotoxicity of lutein on HepG2 2.2.15 cells. The cell viability was evaluated by MTT assay. The data are presented as mean ± SD ($n = 3$).

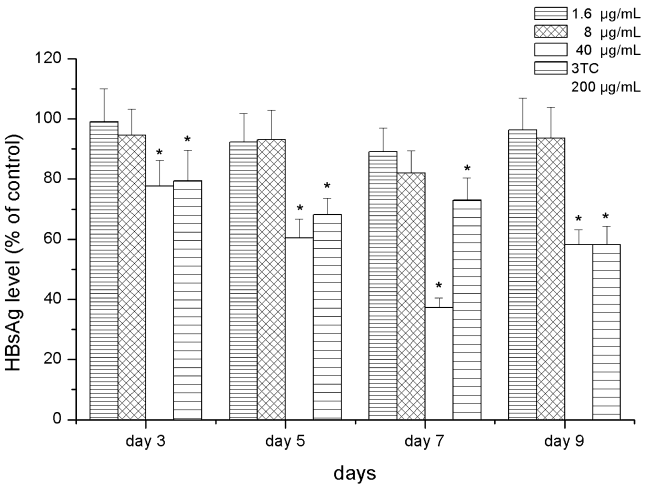

Figure 2. The effect of lutein on HBsAg secretion *in vitro*. The data are presented as mean ± SD ($n = 3$). * $p < 0.05$ compared with the no drug group.

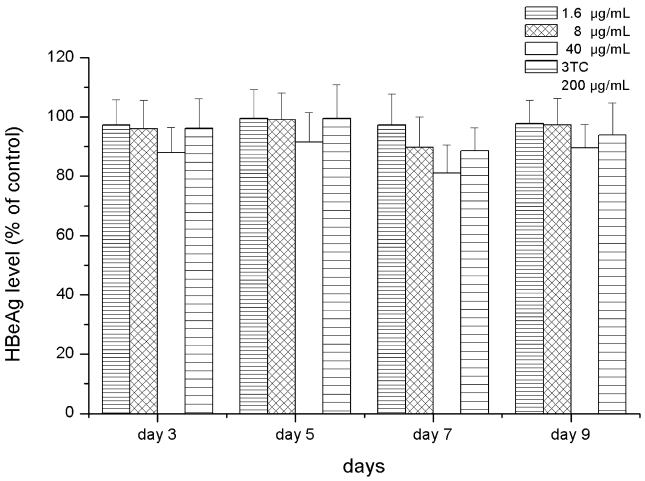

Figure 3. The effect of lutein on HBeAg secretion *in vitro*. The data are presented as mean ± SD ($n = 3$).

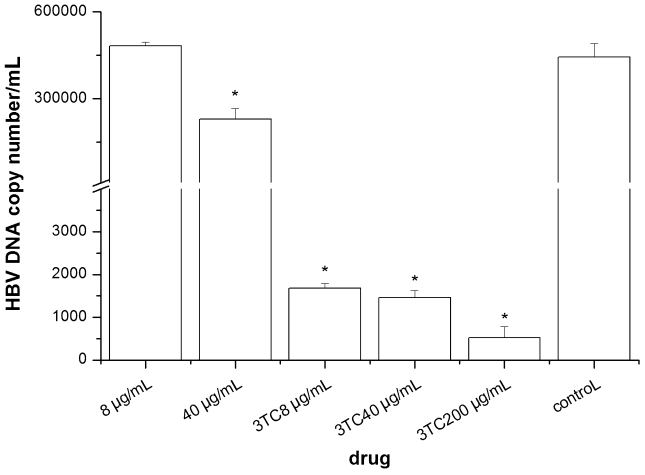

Figure 4. The effect of lutein on HBV DNA replication. Extracellular HBV DNA levels were quantified by real-time PCR. The data are presented as mean ± SD ($n = 3$).* $p < 0.05$ compared with the no drug group.

Table 1. Inhibitory effects of lutein on HBV transcription *in vitro*

	Luciferase activity	
HBV promoter	Lutein (40 μg/mL)	Control
Xp	17.89 ± 3.29	14.90 ± 0.83
S1p	1.76 ± 0.57	1.92 ± 0.50
S2p	119.47 ± 18.75	96.81 ± 5.36
Cp	217.43 ± 33.09	226.03 ± 28.28
Fp	18.45 ± 3.4[a]	37.60 ± 2.88

[a] $p < 0.05$ compared with the no drug group.
The data are presented as mean ± SD ($n = 3$).

Inhibitory effects of lutein on HBV transcription *in vitro*

To examine the effect of lutein on HBV promoter activity, five plasmids were constructed (pCp-Luc, pXp-Luc, pS1p-Luc, pS2p-Luc and pFp-Luc) containing the promoters of four different HBV transcripts and full-length HBV followed by the luciferase reporter gene. After transient transfection of these plasmids into HepG2 cells and lutein treatment, the viral promoter activity was examined by luciferase reporter assay. As shown in Table 1, lutein inhibited the activity of full-length promoter significantly ($p < 0.05$).

DISCUSSION

Although several pharmacological strategies are currently being implemented to treat affected patients, no satisfactory antiviral therapy against HBV infection has yet been fully developed. Thus, it has become urgent to find new and effective anti-HBV drugs. The present study investigated the anti-HBV activity of lutein in stably HBV-transfected HepG2 2.2.15 cells, which can continuously produce complete virion particles of HBV and a high level of viral proteins (Sells *et al.*, 1987). It was found that lutein could decrease the extracellular HBV DNA levels and the secretion of HBsAg significantly. These results demonstrated for the first time that lutein possesses potent inhibitory activity against HBV gene expression and replication *in vitro*.

Several prospective studies have uniformly shown that the HBV DNA level is associated with an increased risk of liver fibrosis, cirrhosis and HCC (Chen CJ *et al.*, 2006; Iloeje *et al.*, 2006; Yang *et al.*, 2002). The HBV viral load is an useful prognostic parameter to evaluate the extent of liver disease in patients with chronic HBV infection (Chen G *et al.*, 2006; McMahon, 2009). In this study, HepG2 2.2.15 cells which were treated with lutein showed suppression of HBV DNA level, implying lutein's potential role to reduce the risk of high HBV DNA level, such as liver fibrosis, cirrhosis and HCC. To further determine whether lutein treatment could decrease the liver damage resulting from HBV infection, study is needed to investigate the antiviral activity of lutein *in vivo*.

The precise modulation of HBV gene expression is essential for replication of the virus, and the expression of HBV is mainly regulated at the transcription initia-

tion level (Moolla *et al.*, 2002; Shamay *et al.*, 2002). Therefore the effects of lutein on the activities of five different HBV promoters: Cp, S1p, S2p, Xp and Fp were examined. These promoters of HBV may act as molecular switches, determining the gene activity. The suppression of 'switch' could further influence the transcription and translation of the HBV gene, resulting in the overall inhibition of viral replication. These studies revealed that lutein is a potent transcriptional inhibitor of viral Fp promoter, but has little effect on other promoter activities in human hepatoma cells. The Fp promoter has a whole region from the upstream enhancer to the pregenomic promoter (Cp), which contains the enhancer I/X promoter (EnI/Xp) and EnII/Cp. It was implied that the inhibitory effects of lutein on Fp promoter may be related to these two enhancers (Choi *et al.*, 1999). Further studies are warranted to clarify how exactly lutein inhibits HBV Fp activity.

In conclusion, lutein could exert anti-HBV activity by decreasing both the level of extracellular HBV DNA and the secretion of HBsAg. It was also found that the antiviral activity of lutein is associated with the inhibition of HBV full-length promoter. Although the exact mechanism remains to be fully elucidated, the data suggest the potential of lutein as an effective anti-HBV reagent with low toxicity.

Acknowledgements

This work was supported by Natural Science Foundation of Hubei Province of China.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

Arbuthnot P, Carmona S, Ely A. 2005. Exploiting the RNA interference pathway to counter hepatitis B virus replication. *Liver Int* **25**: 9–15.

Chen CJ, Yang HI, Su J *et al.* 2006. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* **295**: 65–73.

Chen G, Lin W, Shen F *et al.* 2006. Past HBV viral load as predictor of mortality and morbidity from HCC and chronic liver disease in a prospective study. *Am J Gastroenterol* **101**: 1797–1803.

Choi BH, Park GT, Rho HM. 1999. Interaction of hepatitis B viral X protein and CCAAT/enhancer-binding protein alpha synergistically activates the hepatitis B viral enhancer II/pregenomic promoter. *J Biol Chem* **274**: 2858–2865.

Harikumar KB, Nimita CV, Preethi KC *et al.* 2008. Toxicity profile of lutein and lutein ester isolated from marigold flowers (*Tagetes erecta*). *Int J Toxicol* **27**: 1–9.

Iloeje UH, Yang HI, Su J *et al.* 2006. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* **130**: 678–686.

Kozuki Y, Miura Y, Yagasaki K. 2000. Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. *Cancer Lett* **151**: 111–115.

Liaw YF. 2002. Therapy of chronic hepatitis B: current challenges and opportunities. *J Viral Hepat* **9**: 393–399.

McMahon BJ. 2009. The natural history of chronic hepatitis B virus infection. *Hepatology* **49**: S45–S55.

Moolla N, Kew M, Arbuthnot P. 2002. Regulatory elements of hepatitis B virus transcription. *J Viral Hepat* **9**: 323–331.

Narisawa T, Fukaura Y, Hasebe M *et al.* 1996. Inhibitory effects of natural carotenoids, alpha-carotene, beta-carotene, lycopene and lutein, on colonic aberrant crypt foci formation in rats. *Cancer Lett* **107**: 137–142.

Park NH, Song IH, Chung YH. 2006. Chronic hepatitis B in hepatocarcinogenesis. *Postgrad Med J* **82**: 507–515.

Sells MA, Chen ML, Acs G. 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* **84**: 1005–1009.

Shamay M, Barak O, Doitsh G *et al.* 2002. Hepatitis B virus pX interacts with HBXAP, a PHD finger protein to coactivate transcription. *J Biol Chem* **277**: 9982–9988.

Stringham JM, Hammond BR, Jr. 2005. Dietary lutein and zeaxanthin: possible effects on visual function. *Nutr Rev* **63**: 59–64.

Ukiya M, Akihisa T, Yasukawa K *et al.* 2006. Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (*Calendula officinalis*) flowers. *J Nat Prod* **69**: 1692–1696.

Yang HI, Lu SN, Liaw YF *et al.* 2002. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* **347**: 168–174.

