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Inhibition of the Replication of Hepatitis B Virus in Vitro by Pu-erh Tea Extracts

Shaobo Pei,[†] Yong Zhang, Hao Xu, Xinwen Chen, and Shiyun Chen*

State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China

ABSTRACT: Hepatitis B virus (HBV) is one of the most widespread viral infections in the world and poses a significant global public health problem. The implementation of effective vaccination programs has resulted in a significant decrease in the incidence of acute hepatitis B. Nevertheless, there is still a need for as many effective anti-HBV drugs as possible. In this study, the role of pu-erh tea extracts (PTE) against HBV was analyzed in vitro by using a stably HBV-transfected cell line HepG2 2.2.15. The MTT assay showed that PTE and its active components (tea polyphenols, theaflavins, and theanine) presented low cytotoxicity. ELISA analysis revealed that PTE effectively reduced the secretion of HBeAg, but any one of the active components alone showed weaker efficacy, suggesting that the anti-HBV activity of PTE might be a synergetic effect of different components. RT-PCR and luciferase assay showed that PTE suppressed HBV mRNA expression while leaving four HBV promoter transcriptional activities unchanged. Fluorescence quantitative PCR results demonstrated that PTE dramatically diminished HBV DNA produced in cell supernatants as well as encapsidated DNA in intracellular core particles. Finally, PTE significantly reduced intracellular reactive oxygen species (ROS) level. This study is the first to demonstrate that PTE possesses anti-HBV ability and could be used as a potential treatment against HBV infection with an additional merit of low cytotoxicity.

KEYWORDS: hepatitis B virus (HBV), pu-erh tea extracts (PTE), anti-viral activity

INTRODUCTION

The hepatitis B virus (HBV) belongs to Hepadnaviridae, and its infections are the most common causes of liver disease worldwide. Over 350 million people worldwide are chronically infected with this virus, which can be transmitted parenterally, sexually, or perinatally.[1] Acute HBV infection occasionally results in fulminant hepatitis and usually progresses to a chronic state, which likely leads to decompensated cirrhosis and hepatocellular carcinoma (HCC).[2] After several decades of HBV infection, liver cirrhosis appears in 30−40% of infected persons and HCC develops in 1−5% of cirrhotic patients.[3] Studies have shown that >50% of the registered cases of HCC are associated with HBV infection.[4,5] Even worse, HCC is the third leading cause of death after of lung and stomach cancers in the world, and each fifth diagnosed tumor in the world is HCC.[6,7] The implementation of effective vaccination programs has resulted in a significant decrease in the incidence of acute hepatitis B. Interferon α and nucleotide/nucleoside analogues are widely used in controlling the progression of chronic hepatitis B. However, the immunomodulator interferon α is less effective in curing HBV infection and has some adverse effects.[8] Nucleotide/nucleoside analogues could selectively inhibit the viral polymerase with reverse transcriptase, but long-term therapy might lead to replication of resistant HBV strains.[9] It is therefore urgent to find new antiviral agents for the treatment of HBV infection.

Tea is one of the healthiest and most popular beverages, offering many health benefits. Compared with green tea (unfermented tea), oolong tea (half-fermented tea) and black tea (full-fermented tea),[10] pu-erh tea is one kind of postfermented tea, which undergoes secondary fermentation and oxidation. Studies have revealed that pu-erh tea has many potential functions, for example, anticancer,[11] antioxidant,[12] antiobesity,[13] antimutagenic, antimicrobial,[14] antiarteriosclerosis,[15] and antihyperlipogenesis.[16] However, the chemical composition in pu-erh tea is complex. It is difficult to isolate pure ingredients for structural and functional characterization.[17] Several components of pu-erh tea have been characterized, for example, tea polyphenols (TP), theaflavins (TF), and theanine. TP, which are also known as catechins, possess several activities such as antivirus infection[18,19] and antioxidation and antitumor actions.[20] TF have also been confirmed to have antioxidation[21] and inhibit SARS-CoV 3C-like protease activity.[17] Theanine, which is a green tea-derived amino acid, has been demonstrated to have anxiolytic effects[22] and protects against virus infection.[23]

The aim of the present study was to investigate the anti-HBV ability of the extracts from pu-erh tea. We also tested the roles of three major components (TP, TF, and theanine) in pu-erh tea on HBV replication. We used a stably HBV-transfected cell line, HepG2 2.2.15. HBV antigens, HBV mRNA, HBV gene transcriptional activity, extracellular HBV DNA, encapsidated DNA in intracellular core particles, and ROS level were detected. Our results indicate that PTE effectively inhibits HBV replication, whereas TP, TF, or theanine alone did not function well as PTE, suggesting that the anti-HBV capacity of PTE might be a combination of several chemicals.

MATERIALS AND METHODS

Preparation of Chemicals. Pu-erh tea extracts (PTE), tea polyphenols (TP, 99.40%), and theaflavins (TF, 61.2%) used in this study were all purchased from Gosun Biotechnologies Co., Ltd. (Hangzhou, China). PTE were dissolved in cell culture medium; TP and TF were dissolved in DMSO. Theanine (99.3%) was purchased from Wuxi

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Table 1. PCR Primers Used in This Study

primer	sequence (5′→3′)
HBV-X-F	CCTTCTTACTCTACCGTTCC
HBV-X-R	GACCAATTTATGCCTACAGCC
β-actin-F	CACCAACTGGGACGACAT
β-actin-R	ACAGCCTGGATAGCAACG
HBV-F	GTTGCCCGTTTGTCCTCTAATTC
HBV-R	GGAGGGATACATAGAGGTTCCTT

Southern Yangtze University Biotech Co., Ltd., and dissolved in cell culture medium. All of the stock solutions were freshly prepared immediately before use and were diluted to different concentrations as desired with cell culture medium.

Component Analysis by HPLC. The analysis of the components of PTE and the proportion of theanine in PTE was performed on an Agilent 1200 series HPLC using a reversed-phase column (5 μm, 4.6 × 250 mm). Theanine and PTE were dissolved in 0.05% trifluoroacetic acid/acetonitrile (95:5). A gradient consisting of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) was applied at a flow rate of 1.2 mL/min as follows: 5% B constant from 0 to 10 min, 5− 100% B linear from 10 to 12 min, and 5% B constant from 12 to 15 min. The UV detector was set at 203 nm.

Cell Line and Culture Conditions. The human hepatoblastoma cell line HepG2 2.2.15, which was stably transfected with four 5′−3′ tandem copies of HBV genome,[24] was used in this study. Cells were maintained in DMEM (Gibco) supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, 500 μg/mL G418, and 10% fetal bovine serum (Gibco) at 37 °C in an incubator with 5% CO_2.

MTT Assay. The cytotoxicity effect of the chemicals to HepG2 2.2.15 cells was detected by evaluating the viability of cells through the MTT assay. For each well of 96-well plates, 4×10^4 HepG2 2.2.15 cells were added and cultured at 37 °C for 24 h. The medium was replaced with fresh medium containing PTE, TP, TF, or theanine of different concentrations at an interval of every 2 days. The control wells contained an equivalent amount of solvent. Six days later, the culture medium was replaced with 0.5 mg/mL MTT (Sigma-Aldrich). After incubation at 37 °C for 4 h, the supernatant was aspirated and the formanzan particles were dissolved by adding 150 μL of DMSO. When the precipitant was completely solubilized, the absorbance at wavelengths of 490 and 630 nm was measured. The inhibition rates (percent) were calculated as 100% × [1 − value of the study wells ($A_{490} - A_{630}$)/value of the control wells ($A_{490} - A_{630}$)]. The concentrations of the chemicals with an inhibition rate of 50% (CC_{50}) were calculated according to Berkson's method.[25] The concentrations below CC_{50} were used in subsequent assays.

Treatment of PTE, TP, TF, and Theanine on HepG2 2.2.15 Cells. For each well of 24-well plates, 1×10^5 HepG2 2.2.15 cells were added and cultured at 37 °C for 24 h. The medium was replaced with fresh medium containing PTE, TP, TF, or theanine of different concentrations at an interval of every 2 days. The control wells contained an equivalent amount of solvent. Six days later, the media and cells were collected and used for further experiments.

Detection of HBsAg and HBeAg in Cell Culture Supernatant. HBsAg and HBeAg in the culture medium were analyzed using a commercial ELISA kit (Kehua Bioengineering Corp., Shanghai, China) according to the instructions. The medium samples collected 6 days after treatments were centrifuged at 2000*g* for 10 min and applied to ELISA. The samples were diluted to appropriate concentrations with PBS buffer before measurement. Inhibition rates (percent) of antigens were calculated as 100% × [1 − value of the study well ($A_{450} - A_{630}$)/value of the control well with drug $A_{450} - A_{630}$)]. The concentrations of the chemicals with an inhibition rate of 50% (EC_{50}) were calculated according to Berkson's method.[25]

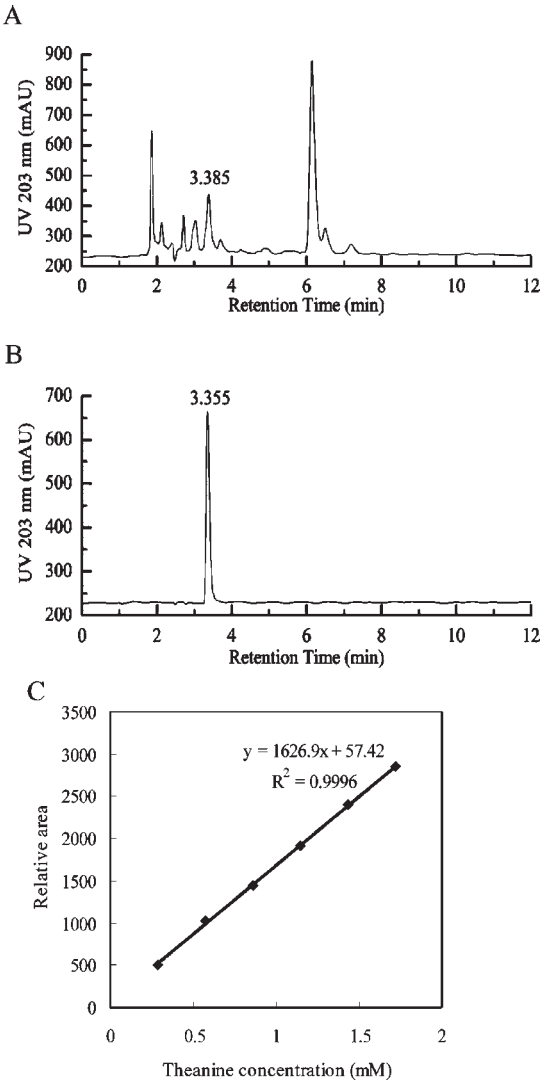

Figure 1. Reverse-phase HPLC analysis of theanine in PTE: (A) PTE sample; (B) theanine standard sample; (C) standard curve of theanine. The chromatograms were generated using a reversed-phase column and a gradient mobile phase as described under Materials and Methods. The detection wavelength was 203 nm.

RT-PCR Analysis of HBV mRNA. Total RNA was extracted by TRIZOL reagent (Invitrogen) according to the instructions. The samples were treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed into cDNA using M-MLV RTase (Promega). The primers used for HBV mRNA analysis were HBV-X-F and HBV-X-R (Table 1), which locate at the X gene of the HBV genome. The primers β-actin-F and β-actin-R (Table 1) were used to amplify the β-actin gene, which was used as an internal reference. The PCR begins with denaturing at 94 °C for 3 min, followed by 30 cycles of 15 s at 94 °C, 15 s at 55 °C, and 15 s at 72 °C.

Luciferase Activity Assay. Five putative HBV gene promoters were respectively inserted into the promoter region of a pGL3 firefly luciferase reporter vector (provided by Prof. Y. Zhu). Each of these recombined plasmids was transfected into HepG2 2.2.15 cells by Fugene HD (Roche) along with the pRL-TK plasmid expressing renilla luciferase as an internal control to normalize transfection efficiency. Three hours later,

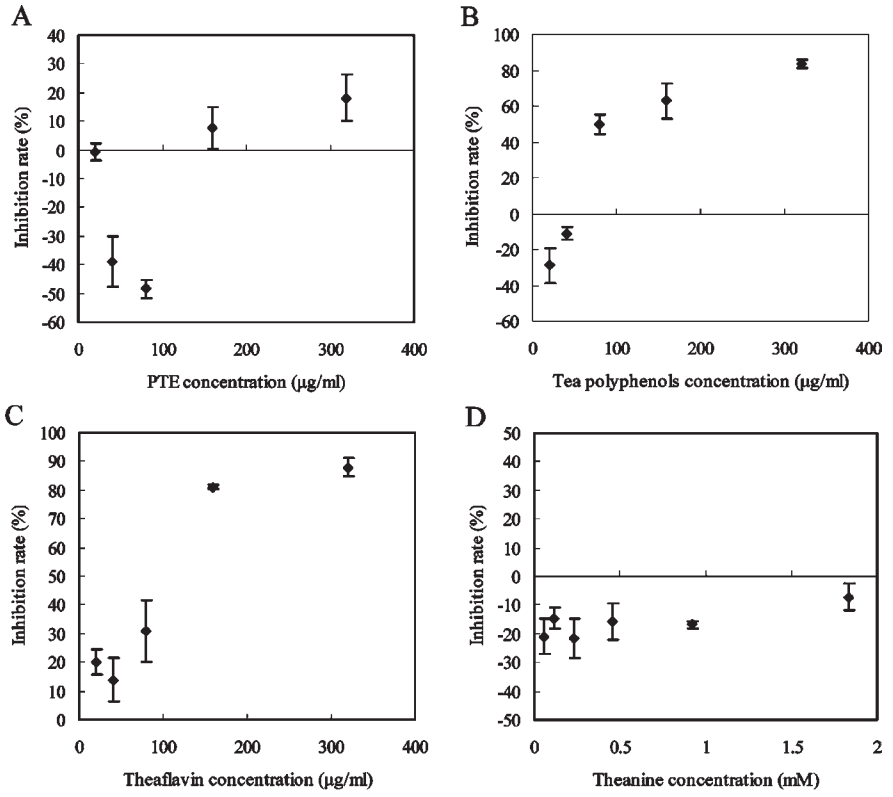

Figure 2. Cytotoxicity analysis of PTE and its ingredients: MTT assays of PTE (A), tea polyphenols (B), theaflavins (C), and theanine (D). Results are expressed as inhibition rate of control (mean ± SD) from six independent experiments.

the medium was replaced with fresh medium containing 320 μg/mL PTE; 48 h later, the luciferase activity was measured using a Dual-Luciferase Reporter (DLR) Assay System (Promega) following the instructions. The reporter activity was calculated as luciferase activity of reporter plasmids in cells treated with PTE compared with that in non-treated cells.

Fluorescence Quantitative PCR. After PTE treatment at different concentration (0, 160, or 320 μg/mL) for 6 days, two forms of HBV DNA were collected and analyzed. Fluorescence quantitative PCR (FQ-PCR) was applied with Syb green fluorescence dye. The primers used for FQ-PCR were HBV-F and HBV-R (Table 1). The FQ-PCR fragment was located at the HBV S gene, and the plasmid containing the cloned HBV genome was used as a standard. To analyze the HBV DNA in cell supernatants, the supernatants were centrifuged at 500*g* for 10 min and incubated at 55 °C for 2 h with an equal volume of lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS, and 400 μg/mL proteinase K). The HBV DNA in supernatants was extracted using buffer-saturated phenol/chloroform (1:1) and incubated at −20 °C for 30 min with 2 volumes of ethanol, 0.1 volume of NaAc (3 M, pH 5.2), and 20 μg tRNA. After centrifugation at 18000*g* for 15 min, the pellets were washed with 70% ethanol and then dissolved in the proper volume of distilled water.

To analyze the HBV encapsidated DNA from intracellular core particles, cells treated with various chemicals in 24-well plates were lysed with 400 μL of buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1% NP-40) on ice for 20 min. The lysate was centrifuged at 18000*g* for 1 min, and the supernatant was incubated at 37 °C for 30 min with 4 μL of 1 M $MgCl_2$ and 4 μL of 10 mg/mL DNase I and then incubated at 55 °C for 2 h with 20 μL of 0.5 M EDTA (pH8.0), 10 μL of 20 mg/mL proteinase K, and 40 μL of 10% SDS. The HBV encapsidated DNA was extracted using phenol/chloroform (1:1) solution and then incubated at −20 °C for 30 min with 0.7 volume of isopropanol, 0.1 volume of 3 M NaAc (pH 5.2), and 15 μg of tRNA. After centrifugation at 18000*g* for 15 min, the pellets were washed with 70% ethanol and dissolved in 15 μL of distilled water. The FQ-PCR program begins with a denaturing step at 95 °C for 5 min and contains 45 cycles of 10 s at 94 °C, 10 s at 57 °C, and 10 s at 72 °C.

Measurement of Intracellular ROS Level. Cells were treated with (160 or 320 μg/mL) or without PTE for 6 days, washed with PBS, and incubated with probe 2′,7′-dichlorfluorescein diacetate (DCFH-DA) for 2 h at 37 °C. Cells were washed with PBS again and then applied to fluorescence measurements with an emission wavelength of 488 nm and an excitation wavelength of 525 nm.

Statistics Analysis. Data analyses were carried out using the Independent Sample *t* test/Univariate program of the SPSS for Windows system. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Cytotoxicity Analysis of PTE and Its Components on HepG2 2.2.15 Cell. Tea polyphenols stably exist in PTE, whereas TF and theanine vary with tea manufacturing sources and procedures.[12,26] For this study, the components of PTE were first analyzed (Figure 1). The HPLC profile showed at least 10 peaks in the PTE sample, and other chemicals with relatively higher proportions than theanine were also found in the PTE sample (Figure 1A). Due to the lack of standard chemicals, we detected only theanine in our PTE samples (Figure 1B). On the basis of the standard curve of theanine (Figure 1C), its content in the PTE sample was calculated as 1.563%.

The MTT assay was then used to analyze the cytotoxicity of PTE and its components, and the results are shown in Figure 2. As shown in Figure 2A, PTE has low cytotoxicity to HepG2

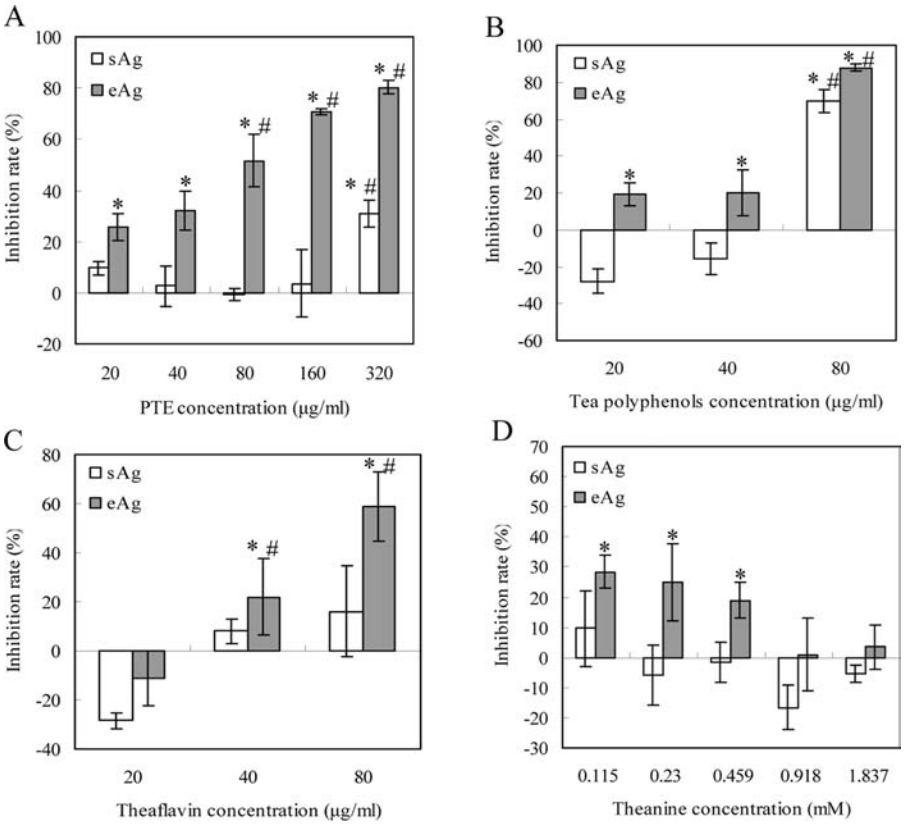

Figure 3. ELISA analyses of HBsAg and HBeAg secreted by HepG2 2.2.15 cells. After treatment with PTE (A), tea polyphenols (B), theaflavins (C), or theanine (D), the culture medium was collected and HBsAg and HBeAg were analyzed by ELISA. Results are expressed as inhibition rates of control (mean ± SD) from three independent experiments. (∗) $P < 0.05$ versus control group; (#) $P < 0.05$ versus last concentration group.

2.2.15 cells. In the MTT assay, the OD value of cells treated with PTE varied, the inhibition rates were sometimes negative, and the CC_{50} of PTE could not be calculated. When the concentration of PTE was at a high concentration of 320 μg/mL, the inhibition rate was only 18.5 ± 8.18%. Under a microscope, little cytotoxicity of HepG2 2.2.15 cells was observed even at a concentration of 320 μg/mL PTE treatment (data not shown). For TP and TF, the CC_{50} values were 134.5 and 142.6 μg/mL, respectively (Figure 2B,C). Theanine also showed no obvious cytotoxicity even at relatively high concentrations of 1−2 mM (Figure 2D).

PTE and Its Components Inhibit the Secretion of HBV Antigens. After treatment with different concentrations of PTE, TP, TF, or theanine, the HBV HBsAg and HBeAg secreted into the medium were detected by ELISA (Figure 3). PTE significantly reduced the secretion of HBeAg in a dose-dependent manner ($P < 0.05$, Figure 3A), and the EC_{50} of HBeAg was 112.2 μg/mL. PTE affected HBsAg slightly with an inhibition rate of 30.82 ± 5.32% at the concentration of 320 μg/mL. Similarly, TP and TF also significantly reduced the secretion of HBeAg ($P < 0.05$, Figure 3B,C), and the EC_{50} values were 52.93 and 70.32 μg/mL, respectively. Theanine at lower concentrations could effectively decrease HBeAg secretion ($P < 0.05$, Figure 3D), whereas little effect on HBsAg secretion was observed. These results suggest that PTE and its components could significantly reduce the secretion of HBeAg. Because PTE acts more effectively than others, we therefore used PTE in our subsequent studies.

PTE Inhibits HBV mRNA Level. To test HBV mRNA level after PTE treatment, RT-PCR analysis was carried out. Because extracted HBV mRNA quality was not good at high PTE concentrations, we used HBV mRNA from cells treated with low PTE concentrations (5−20 μg/mL). As shown in Figure 4, at 6 days after the treatment of PTE on HepG2 2.2.15 cells, the HBV mRNA level was decreased compared to untreated control. This result indicates that the addition of PTE suppressed the transcription of HBV genome.

PTE Regulates Transcriptional Activity of HBV Genes. A Dual-Luciferase Reporter (DLR) Assay System (Promega) was further applied to investigate whether PTE influenced the transcriptional activity of the HBV genes. Four HBV gene promoters were respectively inserted into the promoter region of a pGL3 firefly luciferase reporter vector and transfected into HepG2 2.2.15 cells, with plasmid pRL-TK expressing renilla luciferase as an internal control to normalize the transfection efficiency. At 48 h after 320 μg/mL PTE treatment, the luciferase activities were measured. Compared with untreated cells, PTE regulates HBV gene promoters with a difference. PTE slightly down-regulates activities of S1 and S2 promoters (Figure 5A,B). For HBV X and C promoters, PTE slightly up-regulates their activities (Figure 5C,D).

PTE Inhibits the Production of HBV DNA in Cell Supernatants and HBV Encapsidated DNA in Intracellular Core Particles. Florescence quantitative PCR (FQ-PCR) showed that PTE dramatically diminishes HBV DNA produced in cell supernatants ($P < 0.05$, Figure 6A). At the concentration of 160 μg/mL, PTE could inhibit 30% HBV DNA production in medium. When the PTE concentration was increased to 320 μg/mL, extracellular HBV DNA was undetectable. For PTE treatment, the EC_{50} of HBV DNA in cell supernatants was 205.8 μg/mL. In addition,

FQ-PCR showed that PTE also efficiently impeded HBV encapsidated DNA in intracellular core particles ($P < 0.05$, Figure 6B). At the concentrations of 160 and 320 μg/mL, PTE could inhibit 50.5 and 51.8% HBV encapsidated DNA production in intracellular core particles, respectively.

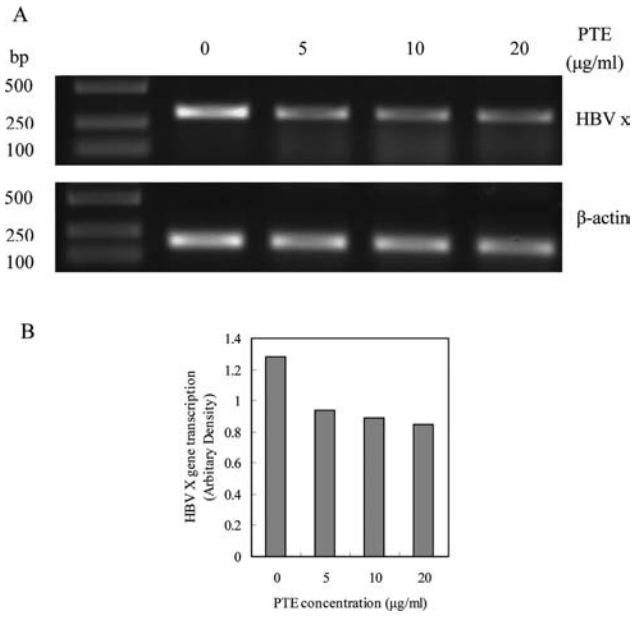

Figure 4. RT-PCR analysis of HBV mRNA in HepG2 2.2.15 cells treated with different concentrations of PTE: (A) representative RT-PCR analysis depicting HBV X gene; (B) relative quantification of HBV X gene transcription. The total mRNA of HepG2 2.2.15 cells was collected at day 6 after treatment with 0, 5, 10, or 20 μg/mL PTE. The results were confirmed by three independent experiments.

PTE Reduces Intracellular ROS Levels. HBV infection will induce intracellular oxidative stress mediated by ROS and cause degenerative diseases.[12,27] We finally tested whether PTE could remove the intracellular ROS in HBV-transfected cells. Intercellular ROS was quantified by fluorescence probe DCFH-DA. At concentrations of 160 and 320 μg/mL, PTE scavenged 40.9 $\pm$ 5.4 and 53.4 $\pm$ 7.8% ROS, respectively (Figure 7). This result reveals that PTE significantly reduces intracellular ROS levels in HBV-infected cells.

DISCUSSION

In this study, we used a stably HBV-tansfected cell line HepG2 2.2.15 to study the anti-HBV effects of PTE in vitro. This cell line is stably transfected with four 5′−3′ tandem copies of HBV genome positioned such that two dimers of the genomic DNA are 3′−3′ with respect to one another,[24] which could synthesize viral nucleic acids[28] and secrete spherical and filamentous forms of HBsAg, core particles, and virions into the culture medium, and has been demonstrated to produce hepatitis in chimpanzees.[29] By analyzing HBV antigen secretion, HBV mRNA level, transcriptional activities of gene promoters, DNA produced in cell supernatants, encapsidated DNA in intracellular particles, and intracellular ROS levels, we confirmed that PTE possesses antiviral activity against HBV.

Our cytotoxicity analysis showed that, at low concentrations, PTE and its components (TP and theanine) have low cytotoxicity to HepG2 2.2.15 cells. This may due to the fact that these chemicals at certain concentrations could increase mitochondrial

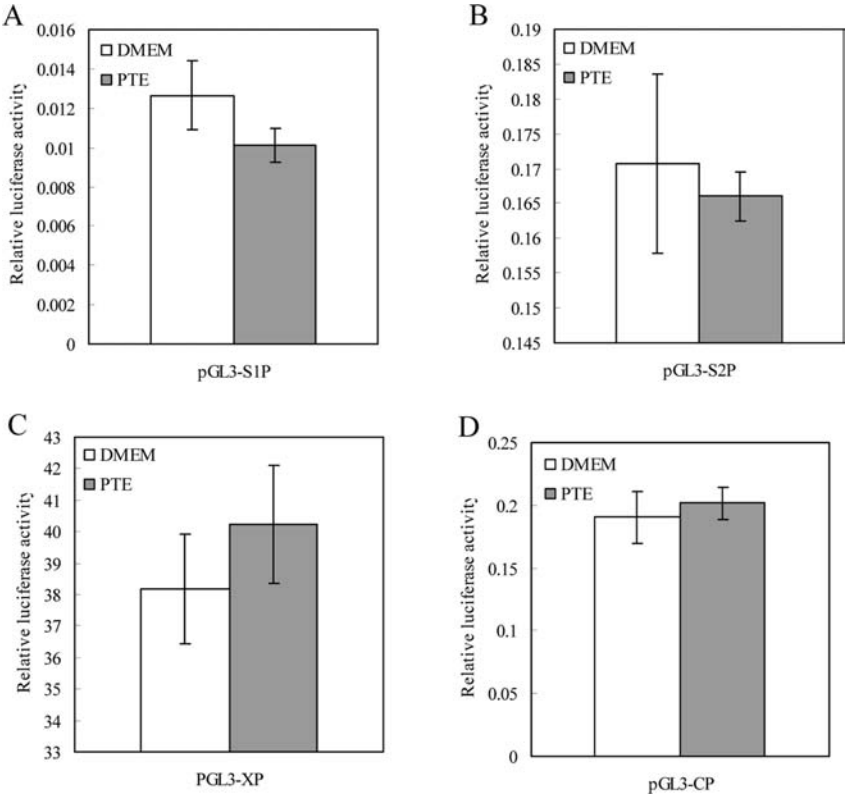

Figure 5. Activity of HBV promoters in HepG2 2.2.15 cells after 320 μg/mL PTE treatment: (A) HBV S1 promoter; (B) HBV S2 promoter; (C) HBV X promoter; (D) HBV C promoter. The results are expressed in "relative luciferase activity" (mean $\pm$ SD) from three independent experiments.

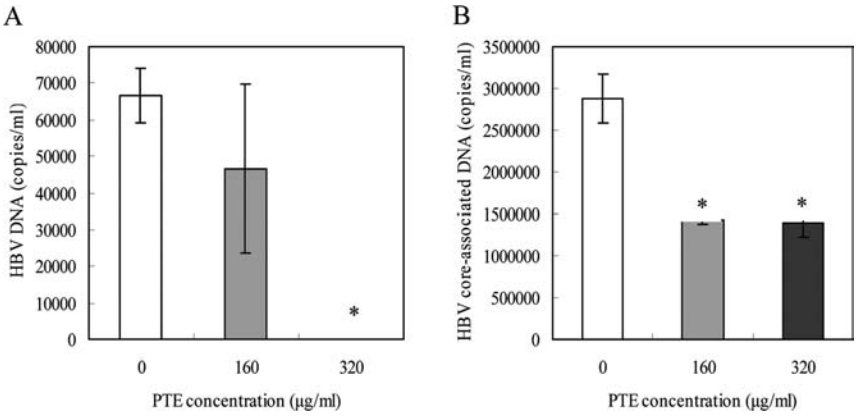

Figure 6. Fluorescence quantitative PCR analysis of HBV DNA and HBV core-associated DNA after treatment with different concentrations of PTE: (A) HBV DNA in HepG2 2.2.15 cell supernatants; (B) HBV core-associated DNA in intracellular core particles. Results are expressed in DNA copies per milliliter of DNA solution (mean ± SD) from three independent experiments. (*) $P < 0.05$ versus non-PTE-treated group.

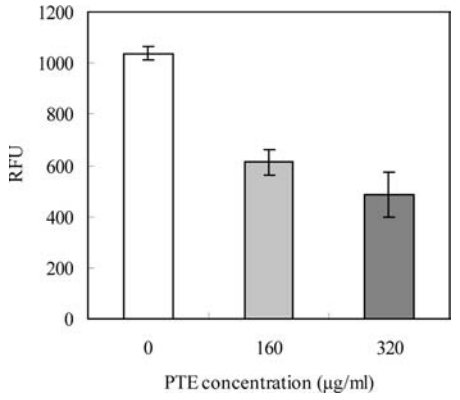

Figure 7. ROS measurement after treatment of HepG2 2.2.15 cells with different concentrations of PTE. Results are expressed in relative fluorescence units (RFU, mean ± SD) from three independent experiments.

succinate dehydrogenase activity and cell proliferation as reported by Hsu et al.[30] The increased cell proliferation may explain why TP and theanine could stimulate HBeAg secretion at low concentrations compared to the controls.

On the basis of the HBV antigen secretion analysis, our results suggest that PTE inhibits HBV antigen production more efficiently than its individual ingredients (TP, TF, and theanine). We suggest two explanations for this. One is that other unknown ingredients in PTE might also have anti-HBV activity. Our HPLC analysis also reveals that the PTE sample contains several chemicals other than the ingredients we have tested. The other explanation is that the anti-HBV effect of PTE might be a synergetic function of different components, which has been demonstrated in a study against HBV replication by others.[31]

In our study, TP could not effectively inhibit HBV replication. It was reported that TP could be a candidate agent for the therapy of HBV infection.[19] There are two reasons for this difference. First, different cell lines were used in these studies, that is, HepG2-N10 and HepG2 2.2.15. The HepG2-N10 cell line was generated by transfecting HepG2 cells with a plasmid that contains a 1.3 unit length of genotype A HBV genome (subtype adw2),[19] whereas the HepG2 2.2.15 cell line was stably transfected with four copies of the HBV genome.[24] The difference of DNA fragments used in cell transfection may lead to different antiviral capacities of the same chemical. Second, the method of TP preparation may also result in antiviral ability changes. Herbal medicines have been used in the therapy of liver disease for a long time. However, they still need standardized manufacture and detailed studies to confirm their efficacy.[32,33]

Our result showed that PTE and its main components inhibit the secreting of HBeAg more effectively than they do HBsAg. HBeAg could be rapidly secreted into the blood and elicits T-cell tolerance. It is reported that split T-cell tolerance between HBeAg and HBcAg and the clonal heterogeneity of HBc/HBeAg-specific T-cell tolerance might have significant implications for natural HBV infection, especially vertical transmission from HBsAg- and HBeAg-positive mothers to neonates.[1,34] Our research indicates that those chemicals, especially PTE, could alleviate host immune tolerance induced by HBeAg during HBV infection.

Theanine at low concentrations could effectively inhibit the secretion of HBeAg in vitro, whereas at high concentrations it did not influence HBV antigen secretion. L-Theanine, also known as γ-glutamylethylamide, is a nonprotein amino acid mainly found naturally in green tea (*Camellia sinensis*). It can pass through the blood−brain barrier and has been demonstrated to have many functions.[22] Because L-theanine has a structure similar to that of glutamic acid and can be hydrolyzed into glutamic acid in the kidney, combined administration of L-theanine with L-cystine enhances immune functions and protects against influenza virus infection in aged mice.[23] Our study is the first to demonstrate that theanine could suppress the secretion of specific HBV antigens with threshold concentration. However, whether theanine can inhibit the replication of HBV needs further studies.

Our fluorescence quantitative PCR analysis leads to the fact that, after the treatment of PTE at the concentration of 320 μg/mL, extracellular HBV DNA was undetectable. We presume HBV DNA is unable to secrete outside the cells. Logically, this disability is not related to the HBV core-associated DNA in intracellular core particles because HBV encapsidated DNA production remained the same when we doubled the concentration of PTE. We propose that high concentrations of PTE may target certain proteins, which will lead to the closing of the HBV particle secretion tunnel as suggested by others;[35,36] the target proteins of PTE are unclear, and further studies are under way to characterize these proteins.

HBV infection induces free radicals, generates oxidative stress, and increases TP53 mutations during the molecular pathogenesis

of HCC.[27] Moreover, compared to HepG2, HBV integration in HepG2 2.2.15 leads to ROS increase and apoptosis.[37] Our results here suggest that PTE could lower the intracellular ROS level induced by HBV integration in vitro, which is consistent with previous data regarding oxidative damage in human fibroblast cells.[12]

In summary, our work demonstrates that PTE could suppress the expression of HBeAg, interfere with HBV transcription, inhibit viral DNA in cell supernatants, reduce viral encapsidated DNA in intracellular core particles, and scavenge intracellular ROS. Pu-erh tea is consumed by people as a daily healthy drink all over the world and has the additional merit of low cytotoxicity.[12] Our study presented here suggests PTE possess potential anti-HBV ability in vitro. Nevertheless, the anti-HBV function in vivo warrants further investigations.

AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +86-27-87199354. E-mail: sychen@wh.iov.cn.

Present Addresses

[†]511 University Village, Salt Lake City, UT 84108, USA.

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ABBREVIATIONS USED

HBV, hepatitis B virus; PTE, pu-erh tea extracts; ROS, reactive oxygen species; HCC, hepatocellular carcinoma; TP, tea polyphenols; TF, theaflavins; FQ-PCR, fluorescence quantitative PCR.

REFERENCES

(1) Rehermann, B.; Nascimbeni, M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat. Rev. Immunol.* **2005**, *5*, 215–229.

(2) Edey, M.; Barraclough, K.; Johnson, D. W. Review article: Hepatitis B and dialysis. *Nephrology* **2010**, *15*, 137–145.

(3) Gurtsevitch, V. E. Human oncogenic viruses: hepatitis B and hepatitis C viruses and their role in hepatocarcinogenesis. *Biochemistry (Moscow)* **2008**, *73*, 504–513.

(4) Block, T. M.; Mehta, A. S.; Fimmel, C. J.; Jordan, R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene* **2003**, *22*, 5093–5107.

(5) Parkin, D. M. Global cancer statistics in the year 2000. *Lancet Oncol.* **2001**, *2*, 533–543.

(6) Parkin, D. M.; Bray, F.; Ferlay, J.; Pisani, P. Estimating the world cancer burden: Globocan 2000. *Int. J. Cancer* **2001**, *94*, 153–156.

(7) Montalto, G.; Cervello, M.; Giannitrapani, L.; Dantona, F.; Terranova, A.; Castagnetta, L. A. Epidemiology, risk factors, and natural history of hepatocellular carcinoma. *Ann. N.Y. Acad. Sci.* **2002**, *963*, 13–20.

(8) Merle, P.; Trepo, C. Therapeutic management of hepatitis B-related cirrhosis. *J. Viral Hepatitis* **2001**, *8*, 391–399.

(9) Kim, J. K.; Lee, H. J.; Lee, Y. J.; Chun, J. Y.; Lee, I. K.; Lim, Y. S.; Suh, D. J.; Ko, S. Y.; Kim, M. H.; Oh, H. B. Direct detection of lamivudine-resistant hepatitis B virus mutants by a multiplex PCR using dual-priming oligonucleotide primers. *J. Virol. Methods* **2008**, *149*, 76–84.

(10) Alcazar, A.; Ballesteros, O.; Jurado, J. M.; Pablos, F.; Martin, M. J.; Vilches, J. L.; Navalón, A. Differentiation of green, white, black, oolong, and pu-erh teas according to their free amino acids content. *J. Agric. Food Chem.* **2007**, *55*, 5960–5965.

(11) Hayakawa, S.; Kimura, T.; Saeki, K.; Koyama, Y.; Aoyagi, Y.; Noro, T.; Nakamura, Y.; Isemura, M. Apoptosis-inducing activity of high molecular weight fractions of tea extracts. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 459–462.

(12) Jie, G.; Lin, Z.; Zhang, L.; Lv, H.; He, P.; Zhao, B. Free radical scavenging effect of pu-erh tea extracts and their protective effect on oxidative damage in human fibroblast cells. *J. Agric. Food Chem.* **2006**, *54*, 8058–8064.

(13) Jeng, K. C.; Chen, C. S.; Fang, Y. P.; Hou., R. C.; Chen, Y. S. Effect of microbial fermentation on content of statin, GABA, and polyphenols in pu-erh tea. *J. Agric. Food Chem.* **2007**, *55*, 8787–8792.

(14) Wu, S. C.; Yen, G. C.; Wang, B. S.; Chiu, C. K.; Yen, W. J.; Chang, L. W.; Duh, P. D. Antimutagenic and antimicrobial activities of pu-erh tea. *LWT—Food Sci. Technol.* **2007**, *40*, 506–512.

(15) Hou, Y.; Shao, W.; Xiao, R.; Xu, K.; Ma, Z.; Johnstone, B. H.; Du, Y. Pu-erh tea aqueous extracts lower atherosclerotic risk factors in a rat hyperlipidemia model. *Exp. Gerontol.* **2009**, *44*, 434–439.

(16) Way, T. D.; Lin, H. Y.; Kuo, D. H.; Tsai, S. J.; Shieh, J. C.; Wu, J. C.; Lee, M. R.; Lin, J. K. Pu-erh tea attenuates hyperlipogenesis and induces hepatoma cells growth arrest through activating AMP-activated protein kinase (AMPK) in human HepG2 cells. *J. Agric. Food Chem.* **2009**, *57*, 5257–5264.

(17) Chen, C. N.; Lin, C. P.; Huang, K. K.; Chen, W. C.; Hsieh, H. P.; Liang, P. H.; Hsu, J. T. Inhibition of SARS-CoV 3C-like protease activity by theaflavin-3,3′-digallate (TF3). *Evidence—Based Complement. Alternat. Med.* **2005**, *2*, 209–215.

(18) Ye, P.; Zhang, S.; Zhao, L.; Dong, J.; Jie, S.; Pang, R.; Li, S. Tea polyphenols exerts anti-hepatitis B virus effects in a stably HBV-transfected cell line. *J. Huazhong Univ. Sci. Technol. Med. Sci.* **2009**, *29*, 169–172.

(19) Xu, J.; Wang, J.; Deng, F.; Hu, Z.; Wang, H. Green tea extract and its major component epigallocatechin gallate inhibits hepatitis B virus in vitro. *Antiviral Res.* **2008**, *78*, 242–249.

(20) Inoue, M.; Kurahashi, N.; Iwasaki, M.; Shimazu, T.; Tanaka, Y.; Mizokami, M.; Tsugane, S. Effect of coffee and green tea consumption on the risk of liver cancer: cohort analysis by hepatitis virus infection status. *Cancer Epidemiol. Biomarkers Prev.* **2009**, *18*, 1746–1753.

(21) Leung, L. K.; Su, Y.; Chen, R.; Zhang, Z.; Huang, Y.; Chen, Z. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr.* **2001**, *131*, 2248–2251.

(22) Weeks, B. S. Formulations of dietary supplements and herbal extracts for relaxation and anxiolytic action: Relarian. *Med. Sci. Monit.* **2009**, *15*, RA256–262.

(23) Takagi, Y.; Kurihara, S.; Higashi, N.; Morikawa, S.; Kase, T.; Maeda, A.; Arisaka, H.; Shibahara, S.; Akiyama, Y. Combined administration of (L)-cystine and (L)-theanine enhances immune functions and protects against influenza virus infection in aged mice. *J. Vet. Med. Sci.* **2010**, *72*, 157–165.

(24) Sells, M. A.; Chen, M. L.; Acs, G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1005–1009.

(25) Berkson, J. Application of minimum logit chi square estimate to a problem of Grizzle with a notation on the problem of 'no interaction'. *Biometrics* **1968**, *24*, 75–95.

(26) Syu, K. Y.; Lin, C. L.; Huang, H. C.; Lin, J. K. Determination of theanine, GABA, and other amino acids in green, oolong, black, and pu-erh teas with dabsylation and high-performance liquid chromatography. *J. Agric. Food Chem.* **2008**, *56*, 7637–7643.

(27) Hussain, S. P.; Schwank, J.; Staib, F.; Wang, X. W.; Harris, C. C. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* **2007**, *26*, 2166–2176.

(28) Sells, M. A.; Zelent, A. Z.; Shvartsman, M.; Acs, G. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *J. Virol.* **1988**, *62*, 2836–2844.

(29) Acs, G.; Sells, M. A.; Purcell, R. H.; Price, P.; Engle, R.; Shapiro, M.; Popper, H. Hepatitis B virus produced by transfected Hep G2 cells

causes hepatitis in chimpanzees. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4641–4644.

(30) Hsu, S.; Bollag, W. B.; Lewis, J.; Huang, Q.; Singh, B.; Sharawy, M.; Yamamoto, T.; Schuster, G. Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 29–34.

(31) Miyata, T. Pharmacological basis of traditional medicines and health supplements as curatives. *J. Pharmacol. Sci.* **2007**, *103*, 127–131.

(32) Modi, A. A.; Wright, E. C.; Seeff, L. B. Complementary and alternative medicine (CAM) for the treatment of chronic hepatitis B and C: a review. *Antiviral Ther.* **2007**, *12*, 285–295.

(33) Dhiman, R. K.; Chawla, Y. K. Herbal medicines for liver diseases. *Dig. Dis. Sci.* **2005**, *50*, 1807–1812.

(34) Chen, M.; Sallberg, M.; Hughes, J.; Jones, J.; Guidotti, L. G.; Chisari, F. V.; Billaud, J.; Milich, D. R. Immune tolerance split between hepatitis B virus precore and core proteins. *J. Virol.* **2005**, *79*, 3016–3027.

(35) Chai, N.; Chang, H. E.; Nicolas, E.; Han, Z.; Jarnik, M.; Taylor, J. Properties of subviral particles of hepatitis B virus. *J. Virol.* **2008**, *82*, 7812–7817.

(36) Shih, C. H.; Li, L. S.; Roychoudhury, S.; Ho, M. H. In vitro propagation of human hepatitis B virus in a rat hepatoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6323–6327.

(37) Niu, D.; Zhang, J.; Ren, Y.; Feng, H.; Chen, W. N. HBx genotype D represses GSTP1 expression and increases the oxidative level and apoptosis in HepG2 cells. *Mol. Oncol.* **2009**, *3*, 67–76.