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Protocatechuic aldehyde inhibits hepatitis B virus replication both in vitro and in vivo

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Abstract

Natural compounds provide a large reservoir of potentially active anti-hepatitis B virus (HBV) agents. We examined the direct effects of protocatechuic aldehyde (PA; derived from the Chinese herb, *Salvia miltiorrhiza*) on HBV replication in HepG2 2.2.15 cell line and duck hepatitis B virus (DHBV) replication in ducklings in vivo. The extracellular HBV DNA, hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) concentrations in cell culture medium were determined by quantitative real-time PCR and ELISA, respectively. DHBV in duck serum was analyzed by dot blot. PA appeared to downregulate the secretion of HBsAg and HBeAg as well as the release of HBV DNA from HepG2 2.2.15 in a dose- and time-dependent manner at concentrations between 24 and 48 μg/mL. PA (25, 50, or 100 mg/kg, intraperitoneally, twice daily) also reduced viremia in DHBV-infected ducks. We provide the first evidence that PA, a novel anti-HBV substance derived from traditional Chinese herb *S. miltiorrhiza*, can efficiently inhibits HBV replication in HepG2 2.2.15 cell line in vitro and inhibit DHBV replication in ducks in vivo. PA therefore warrants further investigation as a potential therapeutic agent for HBV infections.

Keywords: Protocatechuic aldehyde; Duck hepatitis B virus; Hepatitis B virus; *Salvia miltiorrhiza*

1. Introduction

The hepatitis B virus (HBV) belongs to the family of hepadnaviruses (hepatotropic DNA viruses). HBV causes acute and chronic infections of the liver and is responsible for 1.2 million deaths annually (Kane, 1995). Approximately, 80% of carriers have different levels of hepatocyte destruction, which may develop into liver cirrhosis and hepatocellular carcinoma (HCC) (Park et al., 2006). Worldwide deaths from liver cancer caused by HBV infection probably exceed 1 million per year (Parkin et al., 1999). Despite the availability of a safe and effective vaccine against hepatitis B, chronic infection with HBV remains a major health problem worldwide. Although anti-HBV drugs now available have improved the quality of the lives of HBV patients, the apparently inevitable development of drug resistance has prompted the search for new anti-HBV agents. Natural compounds, because of their structural diversity, provide a large opportunity for screening anti-HBV agents with novel structure and mechanism of action.

Salvia miltiorrhiza (SM) is an herb often used in popular folk medicine in China for treating liver disease. SM is considered to be one of the most highly recommended and widely accepted medicines for treating hepatitis B in China (Wang, 2000). Consequently it is of interest to investigate whether SM is effective against HBV. A stably HBV-transfected HepG2 2.2.15 cell line was derived from hepatoblastoma HepG2 cells (Sells et al., 1987, 1988), and has been a useful "in vitro" model for evaluation of novel anti-HBV drugs (Korba and Milman, 1991). Furthermore, the DHBV duck model represents a suitable system for the study of in vivo activity of anti-HBV agents as well

Abbreviations: DHBV, duck hepatitis B virus; HBV, hepatitis B virus; PA, protocatechuic aldehyde; RT, reverse transcription; 3TC, lamivudine; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; pgRNA, pregenomic RNA

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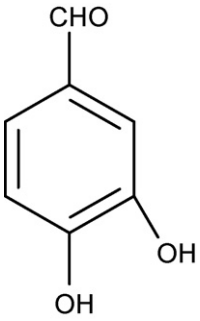


Fig. 1. Chemical structure of protocatechuic aldehyde (PA).

as their toxicity (Mason et al., 1980; Seignères et al., 2001, 2003).

In this study, we isolated and characterized a functionally unique anti-HBV substance, protocatechuic aldehyde (PA, Fig. 1), from SM. PA showed a strong inhibitory effect on the expression of HBsAg, HBeAg, and HBV DNA in HepG2 2.2.15 cells without an effect on the viability of the cells. We also examined the antiviral activity of PA against DHBV replication in chronically infected ducks in a placebo-controlled, dose-ranging study. PA was found to be a remarkably potent and fast-acting antiviral agent against DHBV replication in vivo in the absence of any obvious signs of toxicity.

This is the first report of the anti-HBV effects of PA, an active substance derived from SM. Our observations suggest that PA deserves further investigation as a potential alternative or complementary anti-HBV agent.

2. Materials and methods

2.1. Cell culture

The HepG2 2.2.15 cell line produced by the Mount Sinai Medical Center, New York, was provided by Chinese Academy of Medical Sciences. The HepG2 2.2.15 cells were routinely cultured in Minimum Essential Medium (MEM; Invitrogen, Gaithersburg, MD) supplemented with 10% (v/v) fetal calf serum (Invitrogen, Gaithersburg, MD) at 37 °C in a humidified incubator at 5% CO_2, and 380 μg/mL G418 in the medium was added to select the HepG2 2.2.15 cell line.

2.2. Treatment of PA and lamivudine

PA was purchased as authentic standard from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). lamivudine (3TC) as a reference compound was obtained from GlaxoSmithKline (Suzhou, China). The confluent HepG2 2.2.15 cells were fed medium containing the indicated concentration of drug, and the corresponding suspension was collected for analysis of the levels of HBsAg, HBeAg, and HBV DNA, in triplicate every 3 days for 9 days. The HepG2 2.2.15 cells as control were washed twice with phosphate-buffered saline (PBS) and refed culture medium every 3 days for 9 days.

2.3. Determination of HBsAg and HBeAg

After incubation with various concentrations of PA at 37 °C in 5% CO_2 for 3, 6, and 9 days, conditioned culture medium was collected. The concentration of HBsAg or HBeAg was detected by an enzyme-linked immunosorbent assay (ELISA) kit (Sino-American Biotechnology Co., Luoyang, China) and quantified relative to a standard curve of serial dilutions of recombinant HBsAg or HBeAg.

2.4. Detection of extracellular HBV DNA

Conditioned medium was collected from HepG2 2.2.15 cells. The quantity of HBV in the media of the studied extracts was detected by real-time PCR kit (DA AN GENE CO., Guangzhou, China) following the instruction manual provided. Amplification and detection were performed with an ICycler (Bio-Rad) Detection System. The program was optimized with denaturation at 94 °C for 2 min followed by 40 cycles of amplification (at 94 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s).

2.5. Cytotoxicity assay by MTS

The cytotoxicity of PA was analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following the instruction manual. For evaluation of cytotoxicity, HepG2 2.2.15 cells were seeded at 5000 cells/well into 96-well culture plates (Costar) in 100 μL culture medium and grown for 48 h to reach approximate confluence conditions. Cells were treated or not treated with PA for 3, 6, and 9 days, and then 20 μL of MTS (Promega, Madison, WI) was added to each well and further incubated in CO_2 incubator at 37 °C for 90 min. The absorbance at 490 nm was read in the VICTOR^{TM} 1420 Multilabel Counter (Wallac, Turku, Finland). Data were calculated as a percentage of negative control cells that were not treated with PA.

2.6. Experimental inoculations of ducklings

Sera from ducks that are congenitally infected with DHBV are the source of DHBV used for inoculation. Duck serum positive for duck HBsAg (DHBsAg) at 1:1000 of dilution was used for infection. Ducklings at 1 day of age were intravenously infected with a 5.7×10^6 viral genome equivalent (VGE, 1 VGE = 3.3×10^6 pg) of DHBV as described by Jilbert et al. (1992). Seven days later, PA was administered (25, 50, or 100 mg/kg, intraperitoneally twice daily) for 10 days. PA was solubilized in isotonic saline solution and administered orally in a liquid diet. The isotonic saline liquid diet was also administered to the animals as negative control. Lamivudine (50 mg/kg) was used as positive control. DHBV DNA was measured at days 0, 5, 10, and 3 after cessation of treatment at 10 days (day p3) by dot blot.

2.7. Dot blot analysis for DHBV DNA

For the measurement of DHBV DNA in the serum, a previously reported method was used with modifications (Yao et al., 2001). Briefly, 50 μL of duckling serum was directly spotted on the nitrocellulose membrane, and DHBV DNA was detected with 5-[alpha-^{32}P] deoxycytidine labeled full-length DHBV genomic DNA. Incorporation of radioactivity was determined by Molecular Dynamics storage phosphor screen cassette (Amersham Bioscience). The phosphor screen was scanned with a Typhoon 9410 scanner (Amersham Bioscience) and quantified by using the ImageQuant (Molecular Dynamics) software.

2.8. Statistical analysis

Results are means ± S.D. of the indicated number of independent experiments. Statistical significance was determined using analysis of variance (ANOVA) and Tukey tests. The threshold of significance was set at $p = 0.05$.

3. Results

3.1. Cytotoxic effect of PA on HepG2 2.2.15 cell viability

We first investigated the cytotoxic effects of the drugs on cell viability in HepG2 2.2.15 cells. The results from the MTS test showed that there was no significant difference of cell viability between PA-treated groups whose concentrations were below 72 μg/mL and control group (Fig. 2). PA inhibited the growth of HepG2 2.2.15 cells at concentrations above 72 μg/mL. The cytotoxicity of PA was measured to determine the treatment concentrations in the HepG2 2.2.15 cell culture system.

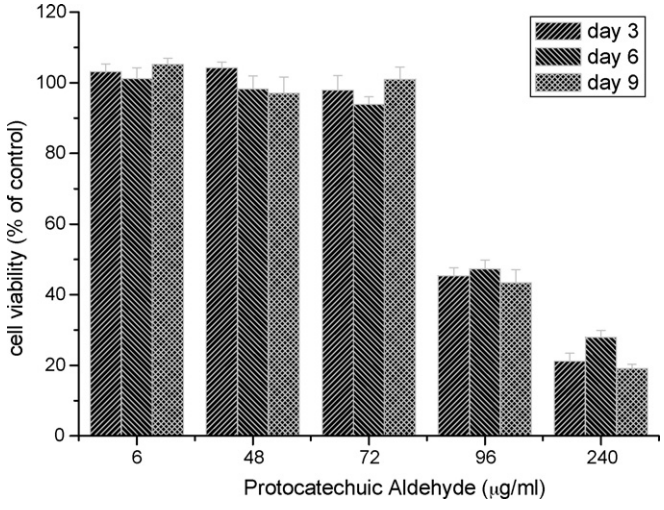
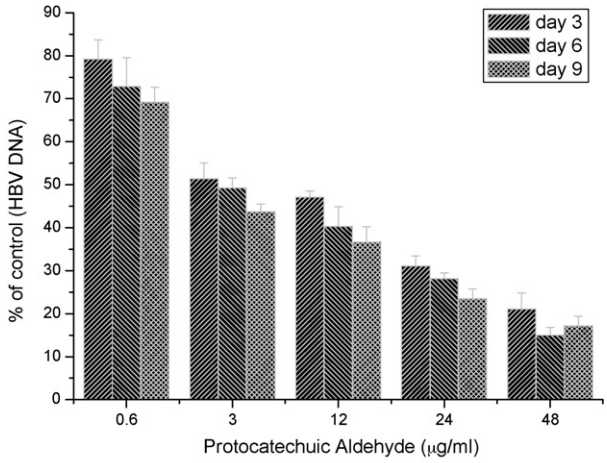


Fig. 2. The cytotoxicity of protocatechuic aldehyde (PA) in HepG2 2.2.15 cells. The inhibition of cell proliferation was measured by MTS method. PA could significantly inhibit the HepG2 2.2.15 cells above 72 μg/mL ($p < 0.05$). The data represent the mean ± S.D. ($n = 4$).



Supplementary information

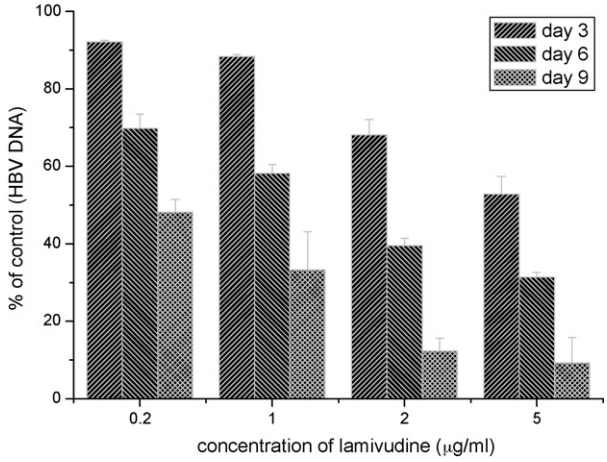


Fig. 3. Effect of protocatechuic aldehyde (PA) on the HBV DNA in HepG2 2.2.15 cells. The amount of HBV DNA in culture media was measured by real-time PCR. Lamivudine (3TC) was used as positive control. PA significantly inhibited the HBV DNA in the HepG2 2.2.15 cells in a dose-dependent manner between 3 and 48 μg/mL ($p < 0.05$). The data represent the mean ± S.D. ($n = 3$).

3.2. Antiviral effect of PA on HepG2 2.2.15 cell

The PA could significantly inhibit the production of HBV DNA dose-dependently (Fig. 3). The IC_{50} of PA to inhibit HBV DNA was 4.17 ± 1.70 μg/mL. The PA could inhibit the expression of HBsAg and HBeAg in a dose dependent manner ($p < 0.05$; Figs. 4–5). The EC_{50} of PA to inhibit HBsAg and HBeAg production was 3.94 ± 1.52 and 2.46 ± 0.38 μg/mL, respectively. The data in this report clearly showed that the inhibitory activity of PA on HBV-induced HBeAg, HBsAg and HBV DNA production was promoted time-dependently ($p < 0.05$).

3.3. Changes in serum concentration of DHBV DNA in the PA treatment study

Serum DHBV DNA was analyzed by dot hybridization (Fig. 6). PA at dosage of 50 and 100 mg/kg significantly inhibited DHBV DNA in duck serum. Densitometric quantitation of the dots revealed a significant decrease in DHBV to 61.9, 51.6,

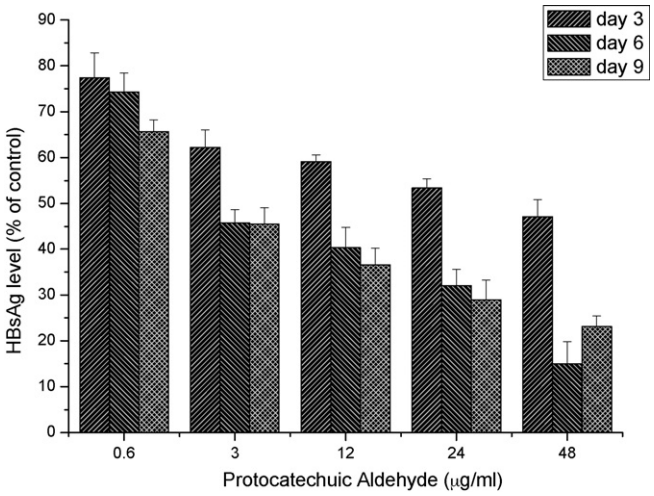


Fig. 4. Effect of protocatechuic aldehyde (PA) on the expression of HBsAg in HepG2 2.2.15 cells. The amount of the expression of HBsAg in culture media was assayed by ELISA. PA significantly inhibited the expression of HBsAg in the HepG2 2.2.15 cells in a dose-dependent manner ($p < 0.05$). The data represent the mean ± S.D. ($n = 4$).

and 64.1% in PA-treated (100 mg/kg) ducks compared with control untreated ducks at days 5, 10, and 3 after the cessation of PA treatment ($n = 6$), respectively. When PA at dosage of 50 mg/kg, the dot signals of the DHBV DNA were reduced to 73.6, 75.1, and 68.6% of the control level at days 5, 10, and 3 after the cessation of PA treatment ($n = 6$), respectively. There were no significant differences in DHBV DNA level between any of the controls.

4. Discussion

Some decades ago, western medicine started to look for novel drugs derived from remedies used in traditional Chinese medicine (TCM) (Wang, 2000; Han, 1988; Dhiman and Chawla, 2005). The vast experience of TCM accumulated over millennia has selected several therapeutic methods, which include the use of herbal therapy (Chen and Chen, 1998). Many natural compounds derived from herbs, including tannic acid, puerarin, baicalin, and artemisinin/artesunate, have been shown to inhibit HBV hepatitis B virus production in HepG2 2.2.15 system (Romero et al., 2005). HepG2 2.2.15 cell contains multiple copies of the HBV genome, which are stably integrated into the host cell genome (Sells et al., 1987, 1988) and is widely used as a useful "in vitro" model for evaluation of novel anti-HBV drugs. SM is frequently used for treating liver diseases in China and oriental countries, and is considered to be the most highly recommended and widely accepted crude medicine for treating liver diseases in China. PA is a pure water-soluble antioxidant compound found in the root of SM (Chen and Chen, 1998). Our results indicated that both HBV DNA and HBeAg, HBsAg were markedly inhibited by PA in HepG2 2.2.15 cell line.

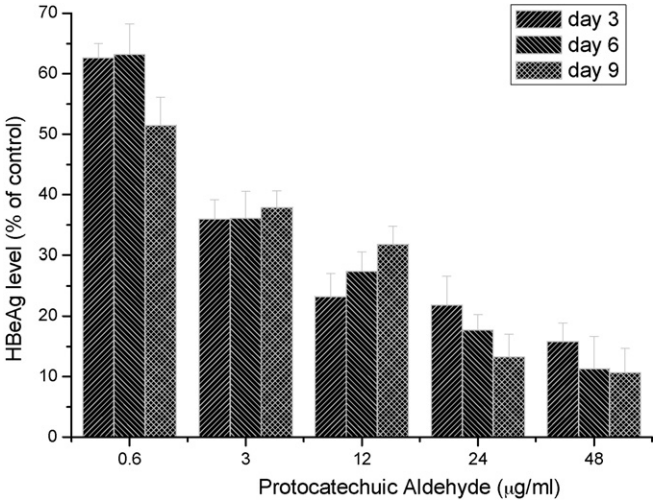


Fig. 5. Effect of protocatechuic aldehyde (PA) on the expression of HBeAg in HepG2 2.2.15 cells. The amount of the expression of HBeAg in culture media was assayed by ELISA. PA significantly inhibited the expression of HBsAg in the HepG2 2.2.15 cells in a dose-dependent manner ($p < 0.05$). The data represent the mean ± S.D. ($n = 4$).

Because infection with HBV is characterized by a narrow species range and tissue tropism, there is no available reliable tissue culture or animal model, other than the chimpanzee (De Meyer et al., 1997). All medical staff and researchers working with HBV must be vaccinated, because of possible nosocomial transmission. Therefore, attention has been devoted to safe animal models such as woodchuck hepatitis virus (WHV) and Duck HBV. These animal viruses have similarities in biology and structural properties to HBV. Furthermore, the duck DHBV model represents a suitable and widely used system for the study of in vivo activity of anti-HBV agents as well as their toxicity (Wang et al., 2002). In this study, we used the HepG2 2.2.15 cell line as well as experimentally infected ducklings to measure the antiviral agent-mediated loss of HBV replication. Data in this report clearly showed that PA inhibits DHBV DNA production in vivo. Our results also indicate that PA show no significant effect on host cells and animals. In contrast to the in vitro analysis of antiviral activity, the evidence from in vivo investigation may be regarded as more convincing. Thus PA seems to be more worth further investigation as an anti-HBV lead structure than other compounds found in TCM which show anti-HBV activity in vitro HepG2 2.2.15 system. These findings might suggest another therapeutic option, other than nucleoside analogues, to manage chronic HBV infection in the future.

Since PA inhibits not only HBV DNA replication but also HBV antigen expression, it is important to note that there might be at least two targets of PA, according to the concept that the regulation of viral replication is quite different from that of the expression of HBV antigen. Inhibition of HBsAg and HBeAg secretion may be achieved at the transcription, translation, or posttranslational level. Although the replication and life cycle of HBV is complex, most known antiviral agents with activity against HBV act at the polymerase or reverse transcriptase (Lee et al., 1989; Price et al., 1989; Doong et al., 1991). PA may cause intracellular retention of HBV DNA by inhibition of HBV polymerase activity. More work is needed,

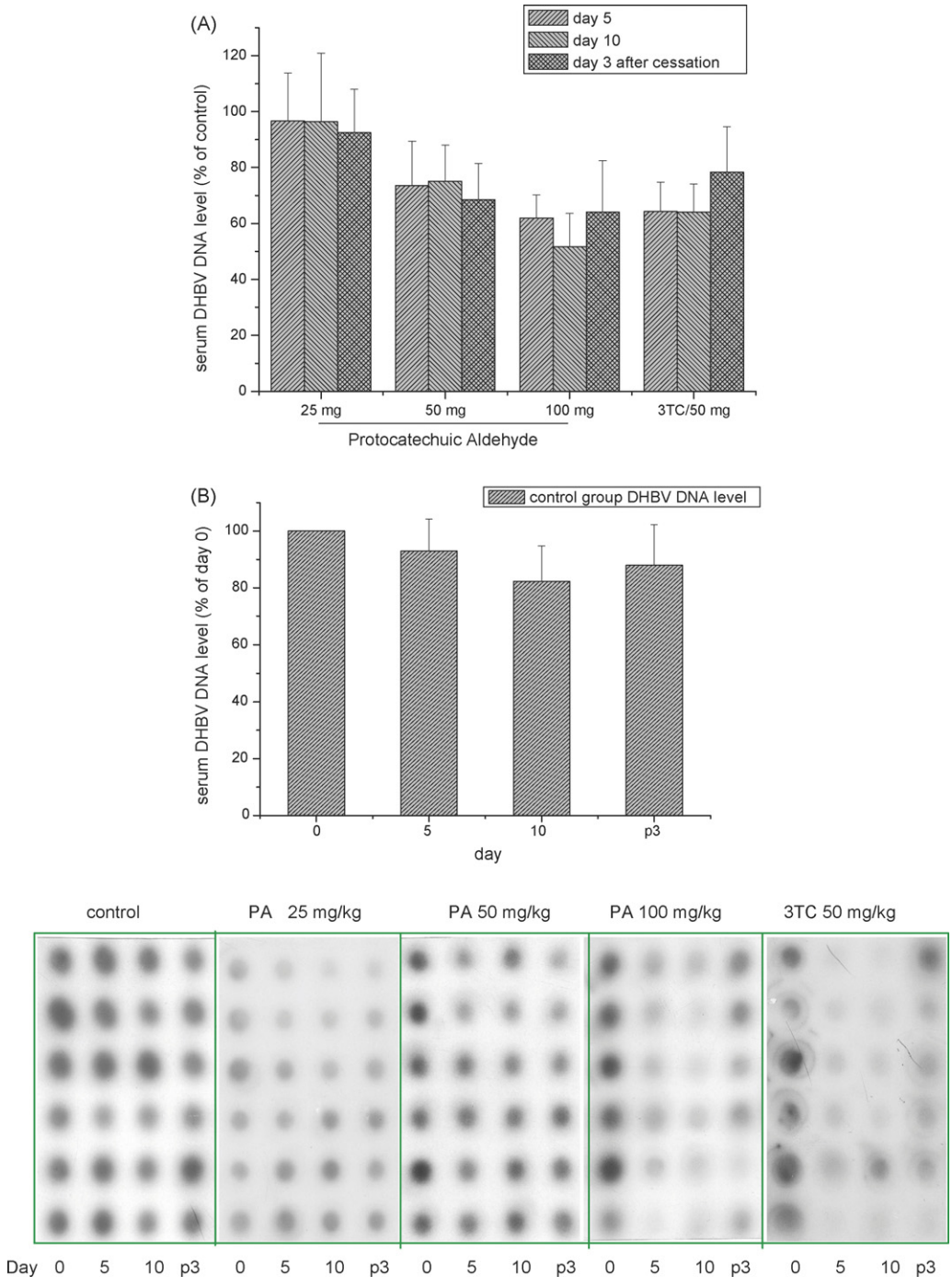


Fig. 6. Dot blots of DHBV DNA in duck serum in the protocatechuic aldehyde (PA) treatment study. Serum DHBV DNA was quantified by the dot blot hybridization method using cloned DHBV DNA as the control and analyzed with a Bio-image analyzer. Lamivudine was used as positive control. Data were expressed as mean ± S.D. ($n = 6$); and were statistically analyzed using Dunnett's multiple comparison test. (A) PA treatment (50, 100 mg/kg) significantly inhibited DHBV DNA in ducks ($p < 0.05$). (B) There were no significant differences between the untreated controls on days 0, 5, 10, and 13 (p3) ($p > 0.05$).

however, to determine the mechanism of anti-HBV activity of PA.

In conclusion, we provide the first evidence that PA, a novel anti-HBV compound derived from traditional Chinese herb SM, can efficiently inhibit HBV replication and the expression of HBeAg and HBsAg in HepG2 2.2.15 cell line and inhibit DHBV DNA replication in ducks in vivo. PA is therefore worthy to be further investigated as an excellent candidate for potential clinical studies.

Acknowledgements

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