Anti-HBV effect of interferon-thymosin α1 recombinant proteins in transgenic *Dunaliella salina in vitro* and *in vivo*

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Abstract. The aim of the present study was to investigate the anti-hepatitis B virus (HBV) effect of interferon (IFN)-thymosin α1 (TA1) in a transgenic *Dunaliella salina* (TDS) system *in vitro* and *in vivo*. The toxicity of TDS in the HepG2.2.15 cell line was assessed using an MTT assay. The effect of TDS on the secretion of HBV early antigen (HBeAg) and HBV surface antigen (HBsAg) in culture supernatants was measured using ELISA. In addition, HBV-DNA was analyzed using quantitative polymerase chain reaction. Drug treatment experiments were performed *in vivo* on ducks congenitally infected with duck HBV (DHBV). The drug was administered once daily for 21 continuous days. Blood was drawn from all ducks prior to treatment, following treatment for 7, 14 and 21 days, and following drug withdrawal for 5 days. Serum DHBV-DNA was determined using quantitative PCR. In addition, the histology of duck liver tissues was assessed using hematoxylin and eosin, and orcein staining. The results demonstrated that TDS suppressed cell viability and HBsAg and HBeAg secretion in HepG2.2.15 cells. Furthermore, the treatment index values for HBsAg and HBeAg following TDS treatment were 2.96 and 3.07 respectively, which were greater than those of the IFN-α treated group. In addition, the DHBV-infected duck model experiments indicated that serum DHBV-DNA levels were significantly decreased in the group of TDS (20 g/kg) following treatment for 7, 14 and 21 days compared with the control group. Following withdrawal of the drug for 5 days, the levels of DHBV-DNA did not relapse in the medium and high dose groups of TDS (10 and 20 g/kg, respectively). Histological analysis of duck liver also demonstrated that TDS and IFN-α treatment alleviated inflammation and HBsAg signals in duck livers. In conclusion, TDS markedly suppresses HBV replication *in vitro* and *in vivo* and its anti-HBV effect is greater than that of IFN-α.

Introduction

Hepatitis B virus (HBV) is a hepatotropic enveloped DNA virus, which causes transient and chronic hepatitis B in humans (1). HBV infection has become a primary public health issue that results in liver cirrhosis and the development of hepatocellular carcinoma (2). In China, early in 2012, ~170 million people are chronically infected with HBV (3). However, effective therapy for patients with HBV is limited owing to a long disease course and easy relapse.

At present, two types of drug are utilized for HBV treatment, including nucleoside analogues and interferon (IFN)-α and its derivatives (4). IFNs are cytokines that exhibit anti-proliferative, antiviral and immunomodulatory activities (5,6). Furthermore, IFN-α serves a key role in the inhibition of viral replication (7). IFN-γ mediates various critical functions by regulating pro-inflammatory, anti-viral and anti-tumor responses (8,9). However, IFNs possess a variety of shortcomings that limit their widespread application, including low response rates, liver decompensation, easy recurrence and numerous side effects, including fever and headache (10). Therefore, enhancing the efficacy of IFN is of vital importance.

The transgenic *Dunaliella salina* (TDS) system has been widely used as a novel bioreactor for expressing exogenous genes (11-13) and has many advantages, including fast growth, low production cost, easy culture, easy transgenic manipulation and a large-scale production of exogenous proteins (13-15). More importantly, the exogenous proteins may also be easily purified to meet the demands of safety and efficiency (9).

HepG2.2.15 cells are derived from the human hepatoblastoma cell line HepG2 and are characterized by exhibiting stable HBV expression and replication within the culture system (16). HepG2.2.15 has been frequently used as a cellular source capable of producing HBV in previous studies (17,18).

The multifunctional cytokine, thymosin α1 (TA1) is a polypeptide hormone with multiple bioactivities that is being clinically trialed for the treatment of HBV and hepatitis C virus (19,20). The present study designed a novel fusion

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interferon (IFN-TA1) combining IFN-α/IFN-γ with TA1 in a TDS system. The aim was to assess the anti-HBV effect of IFN-TA1 in TDS *in vitro* and *in vivo*. The HepG2.2.15 cell line and DHBV-infected duck model were utilized to evaluate the anti-HBV activity of IFN-TA1 in TDS.

Materials and methods

Human tissues. Human liver tissues from 13 patients (6 male, 7 female; age, 31-46) with liver hemangioma receiving liver surgery were obtained from September 2011 to May 2014 in the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Patients were included in the present study if they exhibited normal biochemical indexes and had no history of hypertension, diabetes, fatty liver disease and other chronic diseases, including liver cirrhosis. Patients were excluded if they received hepatotoxic drugs, smoked and consumed alcohol in the first 3 months prior to surgery. The use of human tissue was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) and all patients gave informed consent prior to enrollment in the present study.

Transformation of IFN-α/IFN-γ fusion gene and TA1 in TDS. The *D. salina* strain. UTEX-1644, was obtained from the Algae Culture Collection at the University of Texas (Austin, USA) and were grown in modified PKS medium (NaCl 87.7 g/l; MgS04 with 7H₂0, 1.2 g/l; CaCl₂, 0.022 g/l; KN0₃ 1.0 g/l; KH₂P0₄, 0.054 g/l, ferric salt solution 2.0 ml/l including Na₂; EDTA with 2H₂0 0.74 g/l; FeCl₃, 6H₂0 0.216 g/l) with a 12-h light-dark cycle under a light intensity of 50 mmol photon $m^{-2}s^{-1}$ at 26°C for 24 h (21). To amplify the IFN-α, IFN-γ and TA1 gene, the total RNA of human liver tissues was isolated using Trizol (Sigma, St. Louis, MO, USA). The IFN-α/IFN-γ fusion gene was generated using splicing by overlap-polymerase chain reaction (PCR) (22). The IFN-α/IFN-γ fusion gene and TA1 gene were then inserted into pUΩ-GUS using restriction enzyme HaeIII (New England BioLabs, Inc., Ipswich, MA, USA) and T4 DNA ligase (New England BioLabs, Inc., Ipswich, MA, USA) to generate pUΩ-IFN-α/IFN-γ and pUΩ-TA1 novel vectors, respectively. Subsequently, these vectors were co-transformed into *D. salina* cells using the glass bead method (21). The individual positive TDS colonies were selected using 3 mg/l of phosphinothricin (Hoechst-Roussel AG, Frankfurt, Germany) at 26°C for 5 days and used for further study after 24 h.

Cell culture. HepG2.2.15 cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were incubated in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and streptomycin, 380 mg/l antibiotic G-418 sulfate (Promega Corportation, Madison, WI, USA) and 1% L-glutamine at 37°C in 5% CO_2.

Cell viability assay. Cells were incubated in a 96-well plate at a density of $5x10^4$ cells per 100 μl at 37°C for 24 h. The cells were then treated with 1,000 IU/ml IFN-α or various concentrations of TDS (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) at 37°C for 5 days.

Untreated cells were utilized as a control. Following treatment, cell viability was measured using an MTT assay as described previously (23). Based on the cell cytotoxicity detected by the MTT assay, the concentrations of TDS (0.4, 0.8 and 1.6 mg/ml) were selected for the following experiments in considerations of the lower toxicity. Furthermore, a treatment index (TI) was used to assess the clinical application prospect of the drug (24): TI <1, toxic, ineffective; TI=1-2, effective, with some toxicity; TI >2, greater effectiveness, with low toxicity.

HBV surface antigen (HBsAg) and HBV early antigen (HBeAg) assay. Viral proteins in the culture medium, HBsAg and HBeAg, from the cells treated with different concentrations of TDS (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) were measured by using HBeAg (cat. no. KA3288) and HBsAg (cat. no. KA0286) ELISA kits (Abnova, Taipei City, Taiwan) according to the manufacturers' protocols.

Quantification of HBV DNA. HBV DNA was detected in HepG2.2.15 cells treated with IFN-α or TDS (0.8, 1.6 and 3.2 mg/ml) using quantitative PCR. Total DNA was extracted from the cell supernatant using the TIANamp Virus DNA/RNA kit (Tiangen Biotech Co., Ltd., Beijing, China) and Wizard® Genomic DNA Purification kit (Promega Corporation). The quantification of HBV DNA copies was performed using the SYBR green premix reagent (Takara Biotechnology Co., Ltd., Dalian, China). Total DNA (2 μg) was used as the template for each quantitative PCR assay. PCR was performed using an ABI 7900 real-time PCR detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 93°C for 2 min, 10 cycles at 93°C for 45 sec and 55°C for 60 sec, and 30 cycles at 93°C for 30 sec and 55°C for 45 sec. GAPDH served as a control gene. The primers utilized for HBV DNA fragment amplification were as follows: Forward primer, 5'-CCTCTTCATCCTGCTGCT-3' and reverse primer, 5'-AAC TGAAAGCCAAACAGTG-3'. GAPDH primers: Forward primer, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse primer, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. Assays were repeated in triplicate and mean quantification cycle values were used to calculate the levels of HBV-DNA using the $2^{-\Delta\Delta Cq}$ method (25). The inhibitory rate was calculated using the formula: Inhibitory rate (%)=[DNA copy (C) control-C sample]/C control x100.

Animals and drug treatment. A total of 3 day-old ducklings (weight, 40-50 g) were purchased from Zhejiang Academy of Agricultural Sciences (Zhejiang, China). The sex of ducklings was not distinguished as prior studies have demonstrated that this does not affect results (26,27). All ducks received *ad libitum* access to standard diet and water, and housed under controlled conditions (temperature, 28-30°C; humidity 56-70%; and a 24-h light cycle) (26,27). After adaptive maintenance for 3 days, 0.5 ml of blood was obtained from each duck for analysis. Only DHBV-positive ducklings were used for the subsequent experiments. Animal handling protocols were approved by the Animal Ethics Committees of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Ducks infected with congenital DHBV were randomly divided into 5 groups (each n=6): An IFN-α group treated
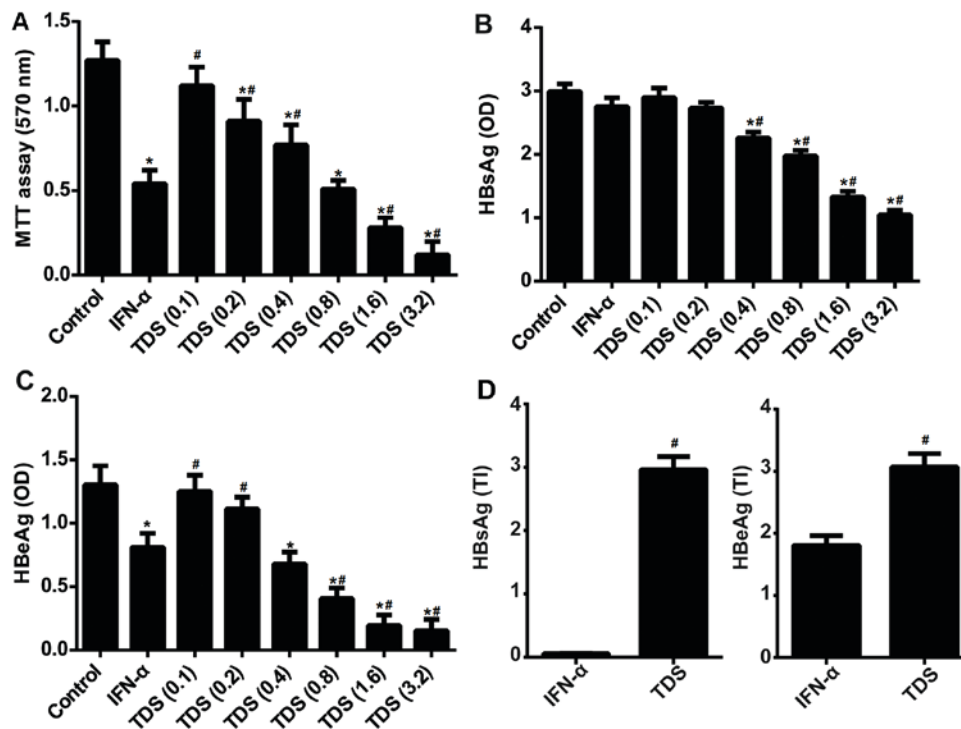
Figure 1. TDS treatment suppressed cell viability and HBV antigen secretion, but promoted TI in HepG2.2.15 cells. Cells were treated with 1,000 IU/ml IFN-α or different concentrations of TDS (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) for 5 days. (A) Cell viability was measured using an MTT assay. (B) HBsAg and (C) HBeAg levels in the culture supernatants. (D) The TI values for HBsAg and HBeAg following TDS and IFN-α treatment in HepG2.2.15 cells. *P<0.05 vs. the control, #P<0.05 vs. IFN-α group. HBV, hepatitis B virus; TI, treatment index; IFN-α, interferon-α; TDS, transgenic *Dunaliella salina*; HBsAG, HBV surface antigen; HBeAg, HBV early antigen; OD, optical density.

with IFN-α (4,000 IU/injections, 500 µl/day; Sigma); 3 groups which received TDS via gastric perfusion at different concentrations (5, 10 and 20 g/kg TDS, respectively); and a control group treated with normal saline. The respective treatments were administered once daily for 21 consecutive days. Blood was drawn from all ducks prior to treatment (T0), following 7 (T7), 14 (T14) and 21 (T21) days of treatment and following withdrawal of the drug after 5 days (P5). The serum samples were separated using centrifugation at 7,000 x g for 15 min at 4°C and stored at -80°C. The levels of DHBV DNA in the serum were detected by quantitative PCR using the SYBR Green real-time PCR Master Mix (Takara Biotechnology Co., Ltd.) with the following primers: Forward primer, 5'-GATACT GGAGCCCAAACC-3' and reverse primer 5'-GGCAGAGGA GGAAGTCAT-3'. GAPDH served as a control gene, forward primer, 5'-CACAGCCACACACGAAGACA-3' and reverse primer, 5'-CCTTAGCCAGCCCCAGTAGA-3'. The thermo-cycling conditions were as follows: 95°C for 1 min, 40 cycles including 95°C for 5 sec, 56°C for 5 sec and 72°C for 25 sec and 40°C for 10 sec. The level of HBV-DNA was calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Histological analysis. Following 21 days of drug treatment, ducks were immediately anesthetized with sodium pentobarbital (Sigma; intraperitoneal injection; 150 mg/kg) and subsequently sacrificed by exsanguination. Liver specimens were collected from the ducks and separated into two sections (1x1 cm). One was fixed with 4% buffered formalin and embedded in paraffin for 24 h at room temperature. The samples were then stained with hematoxylin and eosin (H&E)

for 15 min at room temperature and examined under an optical microscope.

The second section was also fixed with 4% buffered formalin for 24 h at room temperature and embedded in paraffin for 24 h at room temperature. Then the samples were stained with orcein for 30 min at 37°C and examined under an optical microscope to detect HBsAg.

Statistical analysis. Data are presented as the mean ± standard deviation and each experiment was performed in triplicate. Data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Statistically significant differences between two groups were detected using Student's t-test and the comparison of multiple groups was performed using one-way analysis of variance followed by a post-hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of TDS treatment on cell viability and HBV antigen secretion in HepG2.2.15 cells in vitro. The effect of the drug treatment on cell viability and HBV antigen secretion in HepG2.215 cells *in vitro* was assessed. As presented in Fig. 1A, TDS had a marked inhibitory effect on cell viability, suggesting that TDS produced a cytotoxic effect on HepG2.2.15 cells. In addition, treatment with TDS (0.4, 0.8, 1.6 and 3.2 mg/ml) resulted in a significant reduction of HBsAg (Fig. 1B) and HBeAg secretion (Fig. 1C). The TI values of TDS for HBsAg and HBeAg were 2.96 and 3.07 respectively in HepG2.2.15 cells, indicating that TDS was effective and
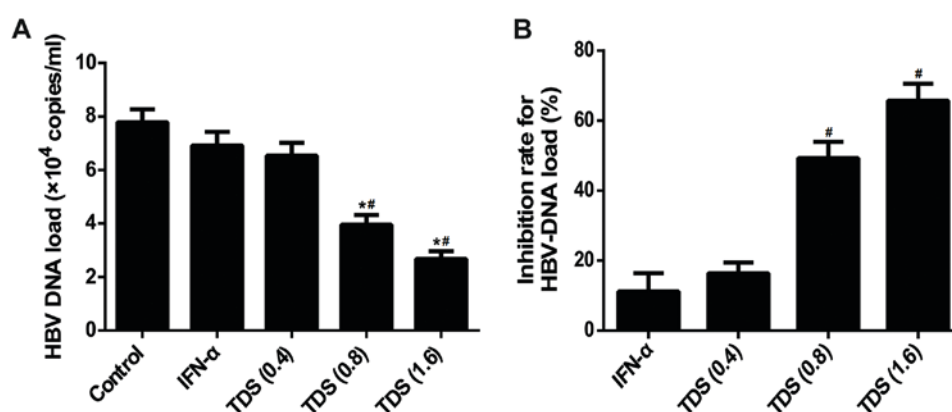
Figure 2. TDS treatment decreased the HBV-DNA load in HepG2.2.15 cell culture medium. (A) HBV DNA levels in the cell supernatant following the administration of different concentrations of TDS (0.4, 0.8 and 1.6 mg/ml) or 1,000 IU/ml IFN-α treatment. (B) The inhibition ratio of IFN-α and different concentrations of TDS on HBV-DNA. *P<0.05 vs. the control, #P<0.05 vs. IFN-α group. HBV, hepatitis B virus; TDS, transgenic *Dunaliella salina*; IFN-α, interferon-α.
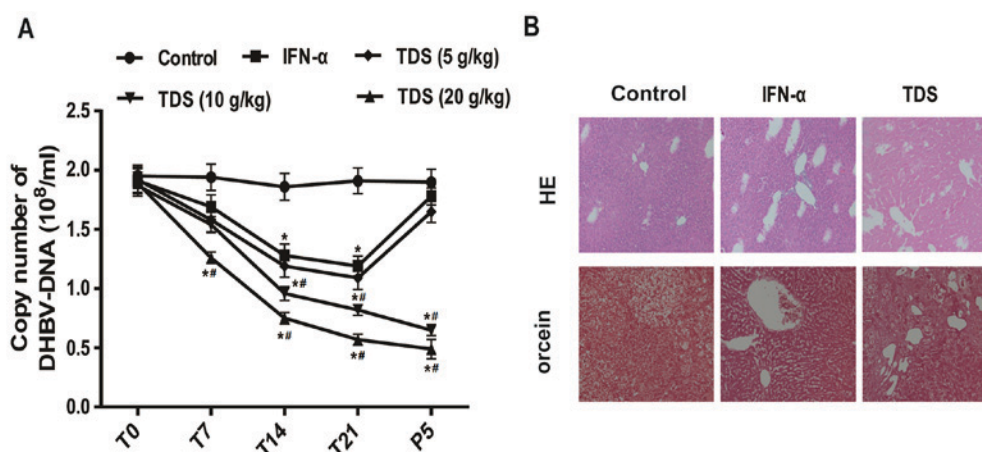

Figure 3. TDS treatment reduced DHBV-DNA levels, inflammation and HBsAg signals in livers. (A) DHBV-DNA levels in the duck serum at different times (T0, T7, T14, T21 and P5) after IFN-α (4,000 IU/injections/d in 0.5 ml) and different concentrations of TDS (5, 10 and 20 g/kg/day) treatment. (B) Representative examples of H&E and orcein staining of the duck livers (magnification, 200x) in control, IFN-α and TDS (20 g/kg) treated groups following treatment for 21 days. *P<0.05 vs. control group, #P<0.05 vs. IFN-α group. DHBV, duck hepatitis B virus; T, treatment; P, drug withdrawal; IFN-α, interferon-α; TDS, transgenic *Dunaliella salina*; H&E, hematoxylin and eosin.

exhibited low toxicity (Fig. 1D). Furthermore, the TI values of TDS for HBsAg and HBeAg were significantly higher than that of IFN-α, which suggests that TDS may be more effective in clinical application.

Effect of TDS treatment on HBV-DNA load in HepG2.2.15 cell culture medium. To further confirm the anti-HBV activity of TDS, the levels of HBV-DNA in the cell supernatant were assessed. The results indicated that, compared with control group, treatment of TDS (0.8 and 1.6 mg/ml) significantly decreased HBV-DNA levels in the culture medium, with inhibition ratios of 49.3 and 65.7%, respectively (Fig. 2A and B). However, no significant difference was observed in the IFN-α group when compared with the control group. The results revealed that TDS treatment suppresses HBV-DNA replication in HepG2.2.15 cells.

Effect of TDS treatment on duck HBV (DHBV)-DNA levels, inflammation and HBsAg in duck livers. Subsequently, the present study assessed the anti-HBV effect of TDS *in vivo*.

As presented in Fig. 3A, duck serum DHBV-DNA levels were significantly decreased in the TDS group (20 g/kg) following treatment for 7, 14 and 21 days compared with that of the control group. Following treatment for 21 days and withdrawal of the drug for 5 days, the levels of duck serum DHBV-DNA in the TDS groups (10 and 20 g/kg) were significantly reduced when compared with the control group. Following drug withdrawal for 5 days, the levels of DHBV-DNA did not relapse in the TDS groups (10 and 20 g/kg). However, relapse following cessation of TDS was observed in the IFN-α and TDS (5 g/kg) groups.

Histological observation was then performed using H&E and orcein staining. The degree of swelling in liver cells was notable and the expression of infiltrating lymphocytes was positive in the control group. However, these results were alleviated following IFN-α and TDS treatment. In addition, HBsAg was observed as brown granules in the control group. However, a decrease in the signals for HBsAg was observed in IFN-α and TDS groups compared with the control group (Fig. 3B). These results demonstrated

that TDS and IFN-α treatment alleviated inflammation and HBsAg in duck livers.

Discussion

IFNs are considered to serve a key role in the control of viral infections (28). A previous study has indicated that interferons suppress HBV replication in transgenic mice that produce a high level of HBV (29). IFN-α has previously been used to treat HBV infections; however, IFN-α treatment generates sustained virological response in a small quantity of patients (30). The incorporation of TA1 into the fusion gene of IFN-α/IFN-γ may be a promising strategy for the development of anti-HBV drugs (31). The present study demonstrated that IFN-TA1 in a TDS model efficiently reduced HBsAg and HBeAg secretion and HBV-DNA replication in HepG2.2.15 cells *in vitro*. Furthermore, TDS treatment suppressed DHBV-DNA levels, inflammation and HBsAg signals in duck livers *in vivo*.

The HBV marker is an essential tool for the assessment of HBV infection (32). Following infection, viral replication occurs inside hepatocytes and consequently HBV DNA, and viral proteins, including HBeAg and HBsAg can be easily detected in serum. The levels of these clinical markers are commonly used to evaluate the disease stage of patients (33,34). Therefore, the inhibition of HBV replication may inevitably reduce the secretion of HBeAg and HBsAg (35). In the present study, the HepG2.2.15 cell line, which contains multiple copies of the HBV genome and is capable of secreting HBV virions into the supernatant, was used as *in vitro* model (16). The results demonstrated that TDS markedly inhibited the levels of HBV DNA and HBsAg and HBeAg in the culture medium of HepG2.2.15 cells. Additionally, TI values for HBsAg and HBeAg were higher in the TDS group than those of the IFN-α group, indicating that TDS treatment enhances the effect of treatment *in vitro*.

DHBV is closely associated with human HBV in regard to its mode of replication, genomic organization and hepatotropism (36). DHBV in its natural host, the duck, has been used as an animal model in a preclinical study of drugs designed for the treatment of HBV (37). The results of the present study demonstrated that TDS was a potent inhibitor of DHBV replication in ducks congenitally infected with DHBV. In addition, DHBV-DNA levels were markedly reduced in the high dosage TDS group (20 g/kg) following treatment for 7, 14 and 21 days and withdrawal of the drug for 5 days compared with that in the control group. The levels of DHBV-DNA did not relapse in the high and medium dosage groups of TDS (20 and 10 g/kg, respectively) following drug withdrawal for 5 days. However, relapse following cessation of TDS was observed in the low dosage TDS (5 g/kg) and IFN-α groups. Additionally, the histological analysis of duck liver confirmed that TDS and IFN-α treatment alleviated inflammatory and HBsAg signals in duck livers. Therefore, these results revealed that TDS strengthens the anti-DHBV effects *in vivo*.

In conclusion, the present study demonstrated that TDS, which produces IFN-TA1 recombinant proteins, effectively inhibited HBsAg and HBeAg secretion and HBV-DNA replication *in vitro* and suppressed DHBV replication and inflammation *in vivo*. The present results indicate that *D. salina* may be used as a bioreactor for the production of IFN-TA1 recombinant proteins. In addition, the anti-HBV effect of TDS is greater than that of IFN-α, which may be an effective antiviral medicine in future treatment of HBV.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ wrote the paper and performed the research; PH and YZ performed the research; XX analyzed the data; SF and CS designed the research. All authors have read and approved this manuscript.

Ethics approval and consent to participate

Animal handling protocols were approved by the Animal Ethics Committees of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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