Cordyceps sinensis attenuates HBx-induced cell apoptosis in HK-2 cells through suppressing the PI3K/Akt pathway

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Abstract. The authors' previous studies demonstrated that the major renal damage from hepatitis B virus infection is HBx-induced apoptosis of renal tubular epithelial cells. *Cordyceps sinensis* is one of the most valuable of traditional Chinese medicines and is extensively used to treat chronic renal diseases. However, there is no research on the potential renal protective effect of *C. sinensis* on HBx-induced apoptosis of renal tubular cells. The protective effect and underlying mechanism of *C. sinensis* were examined using a renal tubular epithelial cell line stably overexpressing HBx. HK-2 cells were stably transfected with pCMV-HBx to establish HBx-overexpression in an *in vitro* cell model and HK-2 cells transfected with an empty vector were generated as a control. The effect of *C. sinensis* on cell proliferation and apoptosis, the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, and the enzyme activity of caspase-3 and caspase-9 was measured. The present study demonstrated that HBx transfection inhibited cell proliferation; increased apoptosis, caspase-3 and caspase-9 activity; and increased the activity of the PI3K/Akt pathway. Treatment with *C. sinensis* attenuated all of these HBx-induced responses. HBx triggered apoptosis and activated the PI3K/Akt signaling pathway in HK-2 cells. *C. sinensis* treatment significantly attenuated the effect of HBx, at least in part by suppressing the PI3K/Akt signaling pathway.

Introduction

Cordyceps sinensis, the caterpillar fungus, is an ascomycete species that is among the most important in traditional Chinese medicine (1). This species has abundant nucleosides, polysaccharides, organic acids, amino acids, peptides, steroids, trace elements and other chemical components (1). *C. sinensis* has numerous therapeutic effects, in that it regulates immune function, intrinsic renal cell proliferation and synthesis of the extracellular matrix and cytokines. Practitioners of traditional Chinese medicine extensively use *C. sinensis* to treat diabetic nephropathy, chronic renal diseases and similar conditions, because of its stimulatory effect on the immune system and its antioxidative activity (2,3).

The authors' previous study found that *C. sinensis* attenuates the disease progression of renal fibrosis by suppressing Bcl-2-associated athanogene 3 induction in a rat model (4). However, no studies have yet addressed the potential protective effect of *C. sinensis* on hepatitis B virus-associated glomerulonephritis (HBV-GN). HBV-GN is a severe health issue in China, the primary cause of secondary renal damage in Chinese children (5-9). HBx is one of the HBV proteins with numerous functions, including activation of the signaling pathways in multiple cell types, regulation of cell proliferation and induction of apoptosis (10-12). The authors' previously demonstrated that HBx-induced apoptosis of renal tubular epithelial cells is one of the major renal injuries from HBV infection (9,13,14).

The integration of multiple pro-apoptotic and anti-apoptotic signals determines whether cells undergo apoptosis (15). Wang *et al* (16) showed that HBx activates the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway in HepG-2 cells. The PI3Ks are a family of enzymes that transduce intracellular signaling to regulate diverse cellular functions, including proliferation, growth, differentiation, survival, motility and intracellular trafficking (17-21).

Previous studies demonstrated that *C. sinensis* attenuates the apoptosis of renal tubular epithelial cells following induction by angiotensin II and ischemia (22-24). However, there is no research on the protective effect of *C. sinensis* on HBx-induced renal tubular cell apoptosis. In this study, a cell line stably expressing HBx was first established, then the role of the PI3K/Akt signaling pathway on HBx-induced renal tubular cell apoptosis was examined. The efficacy and mechanism of *C. sinensis* attenuation of HBx-induced apoptosis in renal tubular cells was subsequently investigated.

Materials and methods

Reagents. Rabbit polyclonal anti-PI3K, rabbit polyclonal anti-phospho-(p-)p85 PI3K, rabbit polyclonal anti-Akt

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and rabbit polyclonal anti-p-Akt (ser473) were obtained from Cell Signaling Technology, Inc. Mouse monoclonal anti-HBx antibody was obtained from Chemicon International; Thermo Fisher Scientific, Inc. Rabbit polyclonal anti-Bcl-2 and rabbit polyclonal anti-Bax were obtained from Santa Cruz Biotechnology, Inc. Cell Counting Kit-8 (CCK-8) was obtained from Nanjing KGI Biological Technology Development Co., Ltd. Caspase-3 and -9 activity assay kit and LY294002 were obtained from Sigma-Aldrich; Merck KGaA. An Annexin V-Fluorescein Isothiocyanate (FITC) and propidium iodide (PI) double staining kit were obtained from Nanjing KeyGen Biotech Co., Ltd. Artificially cultured *C. sinensis* extract [trade name Corbrin capsule (Bailing Jiaonang)] was provided by Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd. and was dissolved in sterile distilled water. The final working concentration was 40 mg/l according to 0.5% (v/v) dilution to the medium (24,25).

Cell culture. A human renal proximal tubular epithelial cell line (HK-2) was obtained from the China Center for Type Culture Collections. HK-2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) containing 4.5 mM glucose, 100 μg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37˚C. Cells were detached using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid. Before treatment, 80-85% of confluent cells were cultured in serum-free media for 12 h to arrest and synchronize the cell cycle. This method can block the cell cycle in the G0/G1 phase and subsequent treatments and cell cycle change analysis based on this method are more convincing (26). In the present study, six repetitions were conducted in each group in every experiment. All experiments were repeated three times. In the *C. sinensis* treatment group, cells were incubated with *C. sinensis* (40 mg/l) for 24 h. In the LY294002 (PI3K inhibitor) treatment experiments, cells received 40 μmol/l LY294002 treatment for 60 min before *C. sinensis* induction (17).

Stable transfection. HK-2 cells were transfected with pCMV-HBx or pCMV-tag2A (empty vector) with Lipofectamine™ LTX and PLUS™ transfection reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 24 h after transfection, the cells were diluted to 1:10 and cultured in growth medium containing G418 (600 μg/ml) for 3 weeks. Stable transfected clones were chosen and maintained in a medium containing 300 μg/ml of G418 for further studies.

Cell proliferation assay. Cell viability was assayed by a colorimetric procedure using CCK-8 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. The absorbance at 450 nm was determined by a microplate reader. The cell suspension was inoculated into 96-well plates (100 μl/well) and incubated overnight. CCK-8 (10 μl) was added to each well and the cells were continuously cultured at 37˚C for 2 h. The optical density value (OD) at 450 nm was determined by a microplate reader. The cellular survival rate was calculated according to the following formulas: Survival rate (%)=(OD value of the test group/OD value of the control group) x100. Inhibition of proliferation rate (%)=(1-survival rate) x100.

Measurement of caspase-3 and -9 activity. HK-2 cells were harvested and centrifuged at 250 x g at 4˚C for 5 min. Cells were washed twice with PBS (pH 7.4) at 4˚C, then re-suspended in 50 μl cell lysis buffer at 4˚C. All steps were performed on ice. The protein concentration was measured using a micro bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Inc.). Each 50 μl cell extract (containing 100 μg protein) was combined with equal volumes of 2X reaction buffer in a microplate and 5 μl peptide substrates of caspase-3 and -9. After incubating overnight in the dark at 37˚C, the samples were examined using a microplate reader at 405 nm. The activity of caspase-3 and -9 was calculated as the absorbance ratio of treated/control samples.

Western blot analysis. Total cell proteins were extracted with radioimmunoprecipitation assay lysate (Sigma-Aldrich; Merck KGaA) containing phenylmethane sulfonyl fluoride. Protein concentration was determined using the BCA kit according to the manufacturer's protocol. Immunoblotting was performed with 50 μg protein, which was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% fat-free milk or 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in TBST [Tris-buffered saline (TBS; pH 7.4) containing 0.21% Tween-20] for 2 h at room temperature. The primary antibodies: HBx (1:1,000; cat. no. MA1-081), t-PI3K (1:1,000; cat. no. 3011T), p-p85 PI3K (1:1,000; cat. no. 4228T), t-Akt (1:1,000; cat. no. 4691S), p-Akt (ser473) (1:1,000; cat. no. 4060S), Bcl-2 (1:500; cat. no. PRS3335), or Bax (1:500; cat. no. SAB4502546) were respectively added and incubated at 4˚C overnight. Next, the secondary antibodies, anti-rabbit IgG (1:5,000; HRP-linked antibody; cat. no. 7074S; Cell Signaling Technology, Inc.) or anti-mouse IgG (1:5,000; HRP-linked antibody; cat. no. 7076S; Cell Signaling Technology, Inc.), were added and incubated at room temperature for 2 h. Bound proteins were visualized using electrochemiluminescence (ECL kit; Pierce; Thermo Fisher Scientific, Inc.) and detected using a DNR BioImaging system 3.2 (DNR Bio-Imaging Systems, Ltd.). β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) was used as an internal control.

Hoechst 33342 staining. Morphological variations in the nuclei of apoptotic cells were observed by staining with HO33342 (Sigma-Aldrich; Merck KGaA). The cell slides were taken out, washed with PBS for three times, fixed with 4% paraformaldehyde at 4˚C for 20 min and washed with PBS again for three times. After HO33342 fluorochrome (5 mg/l) was added, the cell slides were incubated for 8 min at 37˚C (protected from light) and rewashed with PBS three times. Observations and imaging were immediately conducted under a fluorescence microscope. A total of 3 sections were selected in each group and 20 fields of vision were randomly selected for each section. The number of apoptotic cells and the total cells in each random field were counted by two independent researchers. Then the statistical analysis was performed.

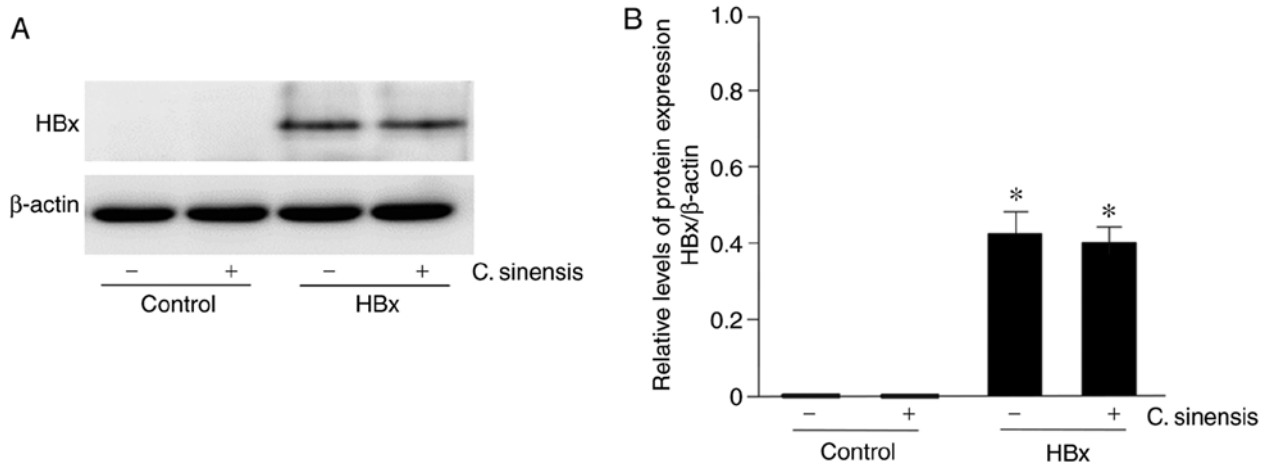

Figure 1. Effect of *Cordyceps sinensis* on expression of the HBx protein in control and HBx-transfected HK-2 cells. (A) Representative western blots of HBx expression in the control group, the control/*C. sinensis* group, the HBx group and the HBx/*C. sinensis* group. In the *C. sinensis* treatment groups, cells were incubated with *C. sinensis* (40 mg/l) for 24 h. (B) Quantitation results of the western blotting analysis. The protein expression level was expressed according to the HBx/β-actin ratio. *P<0.05 vs. the respective control group.

Finally, the equation (apoptotic cells/total cells x100%) was used to indicate the apoptotic rate.

Flow cytometry analysis. The percentage of apoptotic cells was determined using an Annexin V-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd.), quantified by flow cytometry and analyzed with BD CellQuest™ Pro (version S7; BD Biosciences). Cells were stained at 4˚C for 30 min according to the manufacturer's protocol and flow cytometry was conducted using a FACScan flow cytometer (Becton-Dickinson, and Company). Cells were divided into four quadrants: Left lower quadrant (Annexin V-FITC$^-$ and PI$^-$, representing normal live cells), left upper quadrant (Annexin V-FITC$^-$ and PI$^+$, representing cells with mechanical damages), right lower quadrant (Annexin V-FITC$^+$ and PI$^-$, representing early apoptotic cells), and right upper quadrant (Annexin V-FITC$^+$ and PI$^+$, representing late apoptotic cells). The results show the sums of early and late apoptosis.

Statistical analysis. All results are expressed as means ± standard deviations from three independent experiments, and data were analyzed with SPSS version 21.0 (IBM, Corp.). Results were compared using analysis of variance (ANOVA). When the ANOVA indicated a statistically significant difference, multiple comparisons were performed using Tukey's. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of C. sinensis in HK-2 cells stably expressing HBx. HK-2 cells stably expressing HBx were first established by transfection with pCMV-HBx. In the present study, the empty vector pCMV-tag2A was utilized as a negative control (Fig. 1). Western blotting confirmed no expression of HBx in control cells; on the other hand, a prominent protein band at 17 kDa, corresponding to the HBx protein, was detected in lysates from cells stably expressing HBx. Treatment with *C. sinensis* had no effect on the expression of HBx (Fig. 1A and B).

C. sinensis attenuates HBx-induced apoptosis. Cell apoptosis analyzed by flow cytometry indicated that *C. sinensis* treatment had no effect on the apoptosis of control cells (3.53±1.28 vs. 4.21±1.34%; Fig. 2A and C). By contrast, *C. sinensis* treatment significantly inhibited the apoptosis of cells in the HBx group (26.11±4.01 vs. 16.42±3.73%; P<0.05). In the control cells, the nuclei of the HK-2 cells showed clear outlines after HO33342 staining. In the HBx groups, nuclear fragmentations were observed and dissolutions and chromatin margination occurred in some nuclei. *C. sinensis* treatment significantly improved the damage described above (Fig. 2B and D).

Upregulated caspase-3 activity (17.72±2.93%) and caspase-9 activity (16.04±2.11%) were found in HBx-overexpressing cells. In addition, *C. sinensis* treatment decreased the activity of caspase-3 by 7.71±1.81% (P<0.05) and caspase-9 by 8.47±1.36% (P<0.05; Fig. 2E).

C. sinensis attenuates HBx-inhibited cell proliferation. The effect of *C. sinensis* on proliferation was examined in HBx-overexpressing cells. The results showed that HBx overexpression inhibited cell proliferation by 25.27±2.14% and *C. sinensis* treatment significantly attenuated this inhibitory effect (inhibition of 15.02±2.96%, P<0.05; Fig. 3).

C. sinensis treatment attenuates HBx-induced activation of the PI3K/Akt pathway. The phosphorylation level of proteins involving in the PI3K/Akt pathway was examined by western blotting analysis (Fig. 4A and B). The results show that HBx-overexpressing cells had significantly higher levels of the phosphorylated forms of these proteins (P<0.05 for all comparisons) and *C. sinensis* treatment significantly attenuated this effect.

Next, western blotting analysis was performed to examine the expression level of Bcl-2 and Bax (apoptosis regulators) in HK-2 cells (Fig. 4C and D). The results indicated that HBx-overexpressing cells had higher levels of Bax and lower levels of Bcl-2 (P<0.05 for both comparisons). However, *C. sinensis* treatment significantly attenuated these effects (P<0.05 for both comparisons).

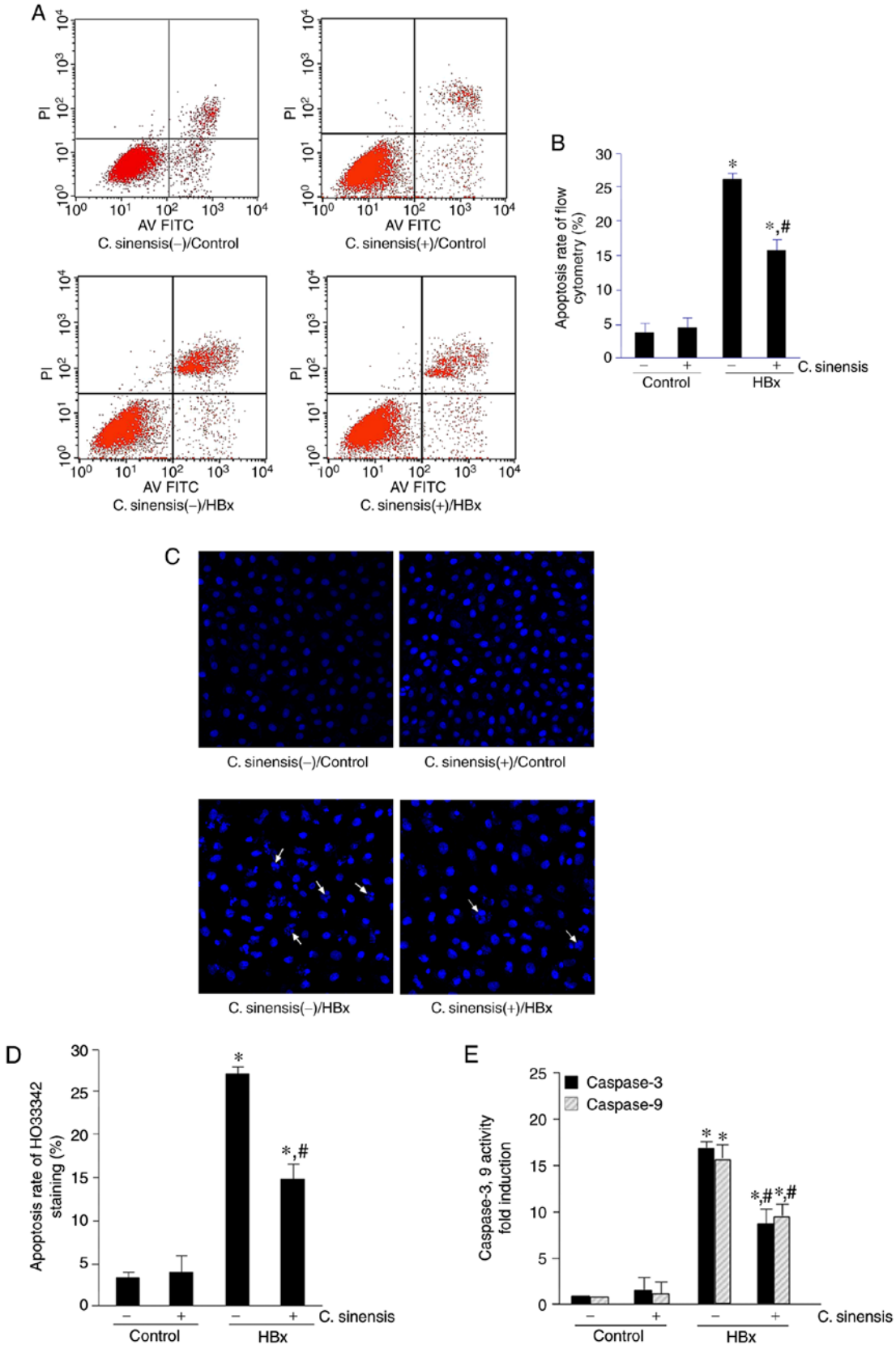

Figure 2. Effect of *Cordyceps sinensis* treatment on apoptosis. (A) Flow cytometry analysis of apoptosis in the control group, the control/*C. sinensis* group, the HBx group, and the HBx/C. sinensis group. In the *C. sinensis* treatment groups, cells were incubated with *C. sinensis* (40 mg/l) for 24 h. Cells were subjected to FITC-conjugated Annexin V and PI staining and analyzed by flow cytometry. The data shown are representative of three separate experiments. The results show the sums of early (the lower right quadrants) and late (the upper right quadrants) apoptosis. (B) HO33342 staining analysis of apoptosis in the control group, the control/*C. sinensis* group, the HBx group and the HBx/*C. sinensis* group. Cell apoptosis was determined by the HO33342 staining assay. The typical apoptotic cell is indicated by the white arrow. Magnification, x200. (C) Quantitation of the flow cytometry results. (D) Quantitation of HO33342 staining results. (E) The effect of *C. sinensis* treatment on caspase-3 and caspase-9 activity in each treatment group. The expression levels of caspase-3 and caspase-9 in the control group were set as 1. The relative expression level of caspase-3 and caspase-9 in the other groups was compared with that of the control group. *P<0.05 vs. the control group $^{\#}$P<0.05 vs. the HBx group. FITC, fluorescein isothiocyanate; PI, propidium iodide.

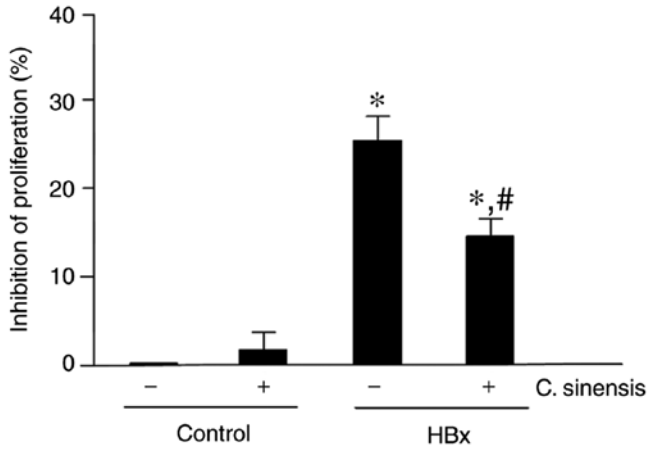

Figure 3. Effect of *Cordyceps sinensis* treatment on the inhibition of cell proliferation. Cell viability in the control group, the control/*C. sinensis* group, the HBx group and the HBx/*C. sinensis* group were determined by the Cell Counting Kit-8 assay. Inhibition of proliferation rate (%)=(1-survival rate) x100. The proliferation inhibition rate of the control group was set to 0 and the proliferation inhibition rate of the other groups was compared with that of the control group. *P<0.05 vs. the control group; #P<0.05 vs. the HBx group.

The effect of the PI3K inhibitor LY294002 on HBx-induced activation of the PI3K/Akt pathway was further investigated (Fig. 5). The results showed that LY294002 treatment alone significantly attenuated the HBx-increased phosphorylated protein level in this pathway (P<0.05 for all comparisons). In addition, treatment with *C. sinensis* and LY294002 together led to further attenuation (Fig. 5A and B). LY294002 had similar effects on the expression of Bax and Bcl-2, and *C. sinensis* treatment also increased this effect (Fig. 5C and D).

Next, the role of the PI3K/Akt signaling pathway in regulating the apoptosis of HBx-overexpressing cells was examined. Consistent with above results, LY294002 attenuated HBx-induced apoptosis and inhibited proliferation, and *C. sinensis* treatment increased this effect (Fig. 6A and B). Similarly, treatment with LY294002 attenuated the HBx-induced changes in the activity of caspase-3 and -9, and *C. sinensis* treatment increased this effect (Fig. 6C). Taken together, the present data indicate that *C. sinensis* treatment attenuates HBx-induced apoptosis and inhibits proliferation by inhibiting the PI3K/Akt pathway in HK-2 cells.

Discussion

Previous studies demonstrated that HBx is involved in multiple cellular processes, including gene transcription, repair of DNA damage, cell cycle, proliferation and apoptosis (27,28). Zhang *et al* (29) reported that HBx inhibited apoptosis. However, other studies reported that HBx enhanced apoptosis by modulating other signaling molecules, such as c-FLIP (30) and Hsp60 (31). These results suggest that HBx promotes or inhibits cell apoptosis, depending on the cell type and the experimental conditions. In this study, it was found that overexpression of HBx significantly increased apoptosis in HK-2 cells. These results are consistent with those of the authors' previous study (13).

C. sinensis is a Chinese herbal medicine commonly used to improve kidney function and to treat renal dysfunction/failure. Specifically, the efficacy of co-treatment with *C. sinensis* in patients with chronic allograft nephropathy is superior to that of immunosuppressive drugs alone and *C. sinensis* has been used to treat chronic allograft nephropathy and in long-term therapy in China (4,32,33). The present study showed that *C. sinensis* treatment attenuated HBx-induced apoptosis. It is well known that PI3K/Akt signaling can regulate cell apoptosis in numerous types of cells (16,34). Therefore, western blotting analysis was used to measure the activity of the PI3K/Akt signaling pathway, such as the phosphorylation of PI3K and Akt in HBx-transfected HK-2 cells. The results showed that HBx transfection activated the PI3K/Akt signaling pathway but *C. sinensis* treatment attenuated the HBx-induced PI3K/Akt signaling. In addition, *C. sinensis* treatment attenuated HBx-induced upregulation of Bax and Bcl-2, both of which play important roles in apoptosis. These data indicate that *C. sinensis* attenuates HBx-enhanced apoptosis in HK-2 cells through suppressing the PI3K/Akt/Bcl-2 pathway.

The P13K/Akt signaling pathway is involved in the regulation of cell proliferation, differentiation, apoptosis and glucose transport. The regulation of apoptosis by the P13K/Akt signaling pathway is very complex. In different cells and environments, its effect can be either anti-apoptotic or pro-apoptotic. To confirm that *C. sinensis* decreases apoptosis by suppressing the PI3K/Akt pathway, a PI3K inhibitor, LY294002, was utilized to suppress PI3K activity in HBx-over expressing cells. LY294002 is mostly known to inactivate Akt, consequently inhibiting cell proliferation and inducing apoptosis. This study indeed observed that LY294002 ameliorated HBx-induced apoptosis by inhibiting the activation of PI3K/Akt. It was found, in several related studies, that under different conditions or in different cell types, LY294002 treatment can reduce cell apoptosis by inhibiting the activation of PI3K/Akt signaling (35,36). This result suggests that the double-sided character of LY294002 in promoting or inhibiting cell apoptosis depends on the experimental cell type and its environmental conditions. In this context, the present results indicate that LY294002 and *C. sinensis* act synergistically to attenuate HBx-enhanced apoptosis. This is the first study to the best of our knowledge to show that *C. sinensis* attenuates PI3K/Akt signaling in stable HBx-overexpressing HK-2 cells.

Cysteine proteases play a critical role in apoptosis regulation. Previous studies indicated that caspase-8, -9 and -10 couple cell death stimuli to the downstream effector caspases, including caspase-3, -6, and -7, to initiate the apoptosis process (37). Once apoptosis is triggered, cellular proteins are cleaved at specific aspartate residues by the effector caspases (38,39). Other studies have reported that HBx overexpression promotes the activation of caspase-3 and -9 (40,41). Thus, activation of caspase-3 and -9 was examined in stable HBx-overexpressing HK -2 cells treated with LY294002 and *C. sinensis*. The current results showed that LY294002 and *C. sinensis* attenuated caspase-3 and -9 activity in these HBx-overexpressing cells in an additive manner. Thus, caspase-3 and -9 activation is required for PI3K/Akt-induced apoptosis in HBx-overexpressing HK-2 cells.

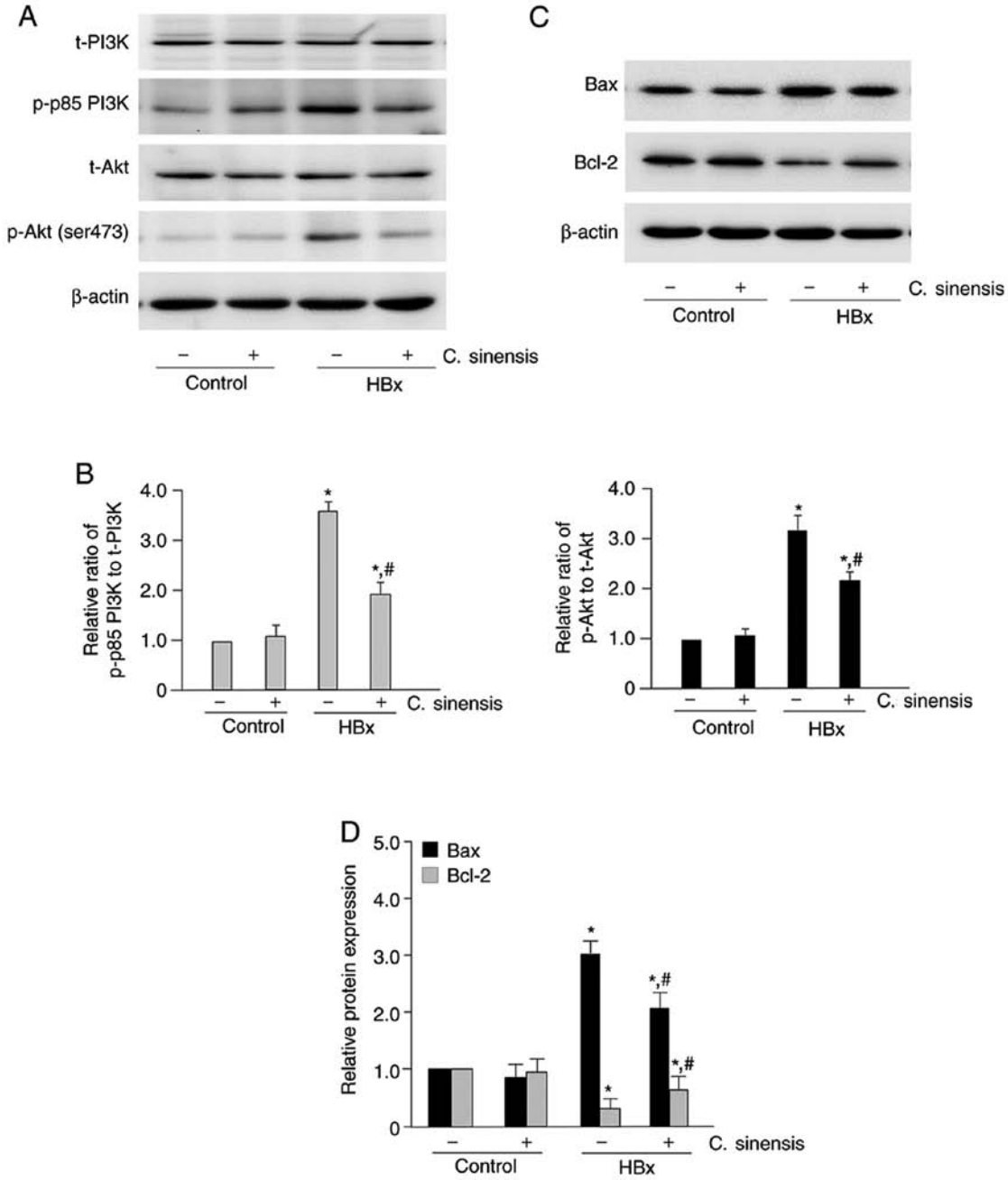

Figure 4. Effect of *Cordyceps sinensis* treatment on the PI3K/Akt signaling pathway and expression of Bax and Bcl-2. (A) Representative western blotting results of p-p85 PI3K, t-PI3K, p-Akt (ser473) and t-Akt in the control group, the control/*C. sinensis* group, the HBx group, and the HBx/*C. sinensis* group. In the *C. sinensis* treatment groups, cells were incubated with *C. sinensis* (40 mg/l) for 24 h. After treatment, total cell lysates were analyzed for the amount of protein of p-p85 PI3K, t-PI3K, p-Akt (ser473) and t-Akt by western blotting. (B) Quantitative results of western blotting. The ratios of p-p85 PI3K to t-PI3K and p-Akt to t-Akt in the control group were set as 1. The relative ratio of p-p85 PI3K to t-PI3K and p-Akt to t-Akt in the other groups was compared with that of the control group. (C) Representative western blotting of Bax and Bcl-2 in the control group, the control/*C. sinensis* group, the HBx group, and the HBx/*C. sinensis* group. (D) Quantitative results of western blotting. The protein expression level of Bax and Bcl-2 in the control group were set as 1. The relative expression level of Bax and Bcl-2 in the other groups was compared with that of the control group. *P<0.05 vs. the control group; #P<0.05 vs. the HBx group. PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; p, phosphorylated; t, total.

In conclusion, the present results demonstrated that HBx induces apoptosis in stable HBx-overexpressing HK-2 cells through PI3K/Akt-Bcl-2 signaling cascades. Treatment with *C. sinensis* significantly attenuated HBx-enhanced apoptosis, at least partly by suppressing the PI3K/Akt-Bcl-2 pathway. Therefore, the current study provides a molecular basis for *C. sinensis* as a treatment option for HBV-GN. Further studies should focus on this regulatory circuit in other *in vitro* and *in vivo* models. In the present study, the relationship between inflammation and HBx-induced apoptosis in HK-2 cells was not investigated. Future studies should seek to characterize the role of inflammation governing this process. In addition, the current team is also working on another cell line, the rat renal tubular epithelial cell NRK52E. Preliminary results showed that *C. sinensis* could also improve the apoptosis induced in these cells by HBx by inhibiting PI3K/Akt signaling. The final data are still being assembled for statistical analysis. It is anticipated that the results will be consistent with those of this study.

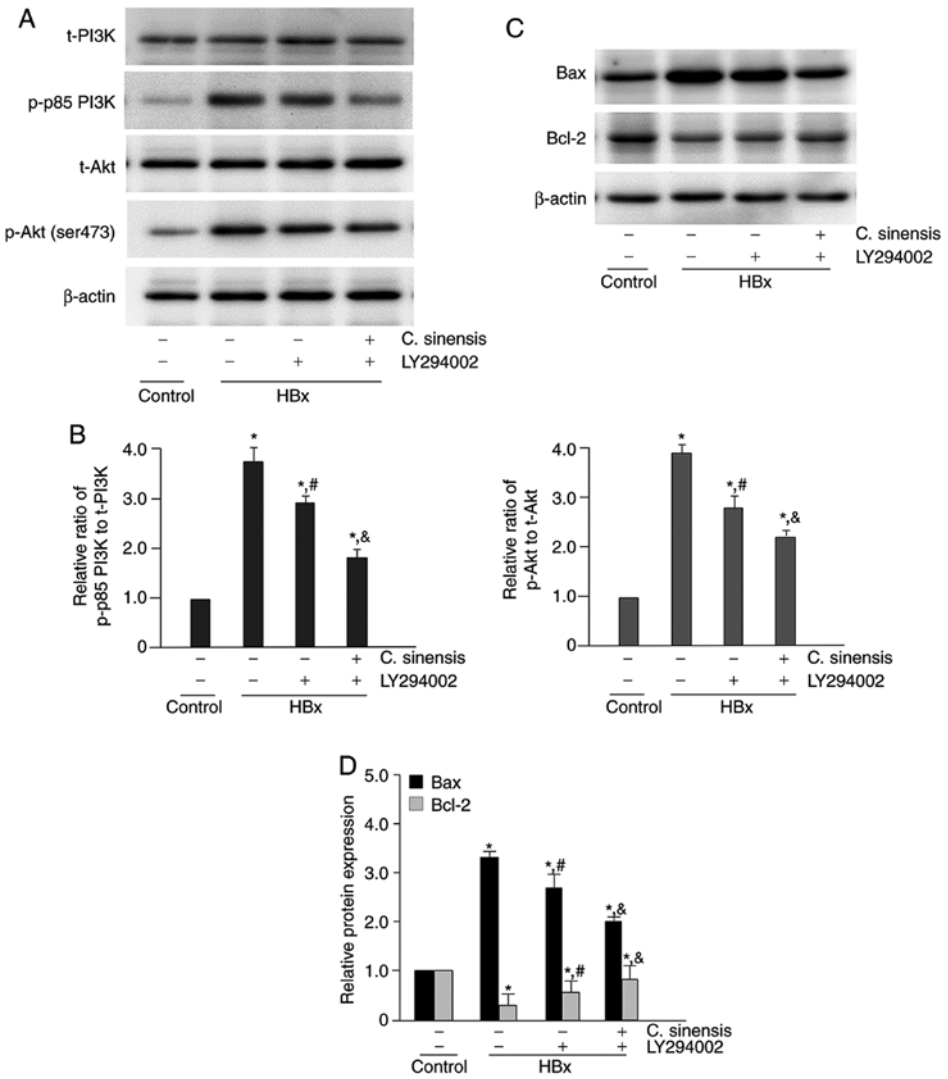

Figure 5. Effect of LY294002 or/and *Cordyceps sinensis* treatment(s) on PI3K/Akt activation and expression of Bax and Bcl-2. (A) Representative western blotting results of p-p85 PI3K, t-PI3K, p-Akt (ser473) and t-Akt in each treatment group. The LY294002 and *C. sinensis* treatment groups received treatment with 40 μmol/l LY294002 (PI3K inhibitor) for 60 min before *C. sinensis* induction. (B) Quantitative results of western blotting. The ratio of p-p85 PI3K to t-PI3K and p-Akt to t-Akt in the control group were set as 1. The relative ratio of p-p85 PI3K to t-PI3K and p-Akt to t-Akt in the other groups was compared with that of the control group. (C) The effect of LY294002 or/and *C. sinensis* treatment(s) on the expression of Bax and Bcl-2. (D) Quantitative results of western blotting. The protein expression level of Bax and Bcl-2 in the control group were set as 1. The relative expression level of Bax and Bcl-2 in the other groups was compared with that of the control group. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the HBx group; $^{\&}$P<0.05 vs. the HBx+LY294002 group. PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; t, total; p, phosphorylated.

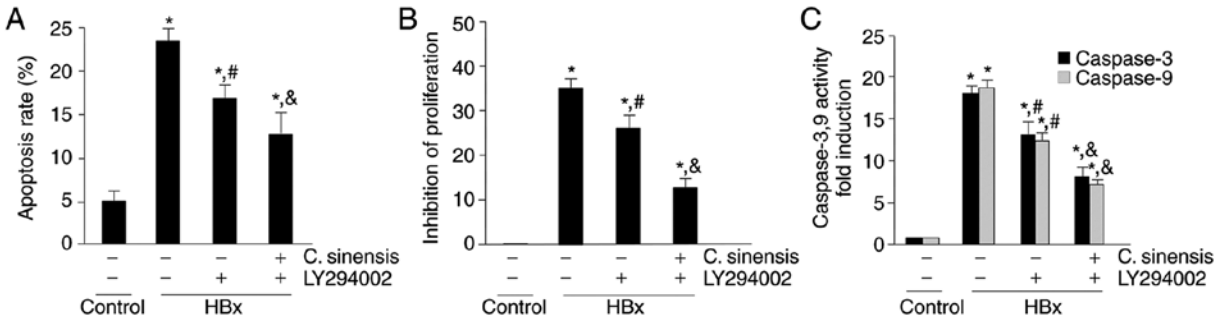

Figure 6. Effect of LY294002 or/and *Cordyceps sinensis* treatment(s) on apoptosis and the inhibition of cell proliferation. (A) Quantitation of the flow cytometry results in each group. In the LY294002 and *C. sinensis* treatment groups, cells received 40 μmol/l LY294002 (PI3K inhibitor) for 60 min before *C. sinensis* induction. After treatment, cells were stained with fluorescein isothiocyanate-Annexin V and propidium iodide. (B) The effect of LY294002 or/and *C. sinensis* treatment(s) on inhibition of cell proliferation was determined by the Cell Counting Kit-8 assay. The proliferation inhibition rate of the control group was set to 0 and the proliferation inhibition of the other groups was compared with that of the control group. (C) The effect of LY294002 or/and *C. sinensis* treatment(s) on caspase-3 and caspase-9 activity. The expression level of caspase-3 and caspase-9 in the control group were set as 1. The relative expression level of caspase-3 and caspase-9 in the other groups was compared with that of the control group. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the HBx group; $^{\&}$P<0.05 vs. the HBx+LY294002 group. PI3K, phosphatidylinositol-3-kinase.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PH and JL were responsible for the cell culture and treatments; CCK-8 experiments and analyses; and for figure preparation. JNM performed the Hoechst 33342 staining and analyses. DW performed the flow cytometry analysis. PH performed the statistical analysis of the data. PH, DW, JNM and JL prepared the images and revised the manuscript. PH and CW performed the western blot experiments. JL prepared the *Cordyceps sinensis* for the experiments. PH and CW performed transfection and cell selection. PH conceived the study. PH and JL wrote the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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