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# Notoginsenoside R1 inhibits hepatitis B virus replication by modulating SIRT1 activity

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**Summary. –** The hepatitis B virus (HBV) infection remains highly prevalent globally. The present study aimed to explore the possible therapeutic effect of notoginsenoside R1, which has attracted considerable attention due to its diverse pharmacological effects, on HBV infection. The HBV-containing hepatocellular carcinoma cell lines, HepG2 and MHCC97H, were used in this study. We first treated the two cell lines with different concentrations of notoginsenoside R1 and subsequently measured the relative levels of HBV DNA, HBV surface antigen, HBV core antigen, and sirtuin 1 (SIRT1) using reverse transcription-quantitative polymerase chain reaction and western blotting. Finally, an HBV hemodynamic replication model was created to test the effect of notoginsenoside R1 on HBV replication. Notoginsenoside R1 inhibited the replication of HBV. This inhibitory effect was mediated through the downregulation of SIRT1 activity. Additionally, the inhibition of SIRT1 activity by silencing its expression or treatment with the SIRT1 inhibitor, selisistat, suppressed HBV replication. Furthermore, our animal experiments demonstrated that notoginsenoside R1 was effective at suppressing HBV replication *in vivo*. Thus, notoginsenoside R1 suppresses HBV replication by downregulating SIRT1 activity *in vitro* and *in vivo*.

**Keywords:** notoginsenoside R1; hepatitis B virus; SIRT1

## Introduction

Hepatitis B virus (HBV) replicates in hepatocytes, resulting in apoptosis and chronic infection (Glebe and Bremer, 2013). In addition to causing hepatic infections, HBV integrates its DNA into the genome of infected hepatocytes, which may contribute to hepatocarcinogenesis (Seeger and Mason, 2015). Although the HBV vaccine is more than 95% effective at preventing HBV infection, the virus is prevalent in many developing nations, in addition to specific populations, such as prisoners and human immunodeficiency-virus-(HIV)-positive patients (Stasi *et al*., 2017). Interferon-α and nucleoside or nucleotide analogs are the primary treatment options for HBV infections (European Association for the Study of the Liver, 2017). However, the development of multi-drug resistance and the occurrence of mutations have been reported for HBV, resulting in treatment failure (Warner and Locarnini, 2014; Zoulim, 2011). Based on the characteristics of HBV, new strategies for HBV treatment have emerged, such as targeting the SLC10A1 protein, using RNA interference to

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**Abbreviations:** HBV = hepatitis B virus; HIV = human immunodeficiency virus; HCC = hepatocellular carcinoma; si = small interfering; HBcAg = hepatitis B virus core antigen; HBsAg = hepatitis B virus surface antigen; HBeAg = hepatitis B virus envelope antigen; pgRNA = pregenomic RNA; SIRT1 = sirtuin 1

suppress HBV replication, and T cell engineering (Testoni *et al*., 2017).

Notoginsenoside R1 is a compound extracted from *Panax notoginseng*. It has an array of pharmacological properties. It exerts protective effects on neurons and cardiomyocytes, has anti-inflammatory effects, and suppresses the development of cancers (Guo *et al*., 2019). Although notoginsenoside R1 has not been reported to possess antiviral properties, *P. notoginseng* has been reported to exert suppressive effects on the replication of viruses. For instance, it inhibits influenza A virus replication by increasing immune activity (Choi *et al*., 2017). Additionally, *P. notoginseng* inhibits the expression of HIV reverse transcriptase (Lam and Ng, 2002). Notably, hepatic microcirculation is ameliorated by notoginsenoside R1 (Chen *et al*., 2008) due to its inhibitory effects on hepatocellular carcinoma (HCC) (Li *et al*., 2020). Therefore, we aimed to investigate the possible inhibitory effect of notoginsenoside R1 on HBV replication.

To the best of our knowledge, the effects of notoginsenoside R1 on HBV replication have not previously been investigated. In the present study, we measured HBV replication *in vivo* and *in vitro* following notoginsenoside R1 treatment. Notoginsenoside R1 was found to inhibit HBV replication by decreasing sirtuin 1 (SIRT1) activity. Our findings provide insights into the management of HBV infections.

## Materials and Methods

*Cells and cell culture.* HepG2 and MHCC97H cells were procured from the American Type Culture Collection (USA). They were cultured in Dulbecco's modified Eagle's medium (Beyotime, China) containing 10% fetal bovine serum (Gibco, USA). The cells were then incubated at 37°C in a humid atmosphere (5% $CO_2$) and either passaged or used for further experiments when cell confluence reached 70–80%.

*Cell transfection.* A small interfering (si) RNA targeting *SIRT1* (si-*SIRT1*) and a negative control siRNA (si-NC) were purchased from GenePharma (China). Lipofectamine 3000 (Invitrogen, USA) was used as the transfection reagent, and transfection was performed based on the manufacturer's protocols.

**Table 1. Primer sequences**

| Gene | Species | Primer sequence (5'→3') |
|---|---|---|
| *SIRT1* | Human | F: TGTGTCATAGGTTAGGTGGTGA |
| | | R: AGCCAATTCTTTTTGTGTTCGTG |
| *GAPDH* | Human | F: GGAGCGAGATCCCTCCAAAAT |
| | | R: GGCTGTTGTCATACTTCTCATGG |
| *SIRT1* | Mouse | F:TGTCTCCTGTGGGATTCCTGACTTC |
| | | R:TGGCTTGAGGGTCTGGGAGGT |
| *GAPDH* | Mouse | F:AATGGATTTGGACGCATTGGT |
| | | R:TTGCACTGGTACGTGTTGAT |

*Reverse transcription-quantitative polymerase chain reaction.* TRIzol reagent (Sigma-Aldrich, USA) was used to obtain total RNA from cells and tissues. mRNA was transcribed into cDNA using the One-Step RT-qPCR probe kit (Yeasen, China) and cDNA was quantified using SYBR green (Roche, Switzerland). The sequences of the primers used in this study are listed in Table 1. The following primers and probes used to amplify pregenomic RNA (pgRNA) were added to the reaction mixture: forward primer, GGTCCC CTAGAAGAAGAACTCCCT; reverse primer, CATTGAGATTCCCGA GATTGAGAT; and probe sequence, TCTCAATCGCCGCGTCGCAGA. PCP (5'-GGTCTGCGCACCAGCACC nt 1796-1813) was used for the detection of HBV *preC* mRNA. *GAPDH* was used as an endogenous control. To calculate the relative mRNA expression levels, were calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

*Cell viability assay.* To perform the Cell counting kit-8 (CCK-8) assay, HepG2 and MHCC97H cells were inoculated into 96-well plates at concentration of $3 \times 10^3$ cells/well. When the cells reached 50% confluence, they were transfected with different doses of notoginsenoside R1 followed by incubation for 72 h. The cells were then subjected to a CCK-8 assay (Dojindo Laboratories, Japan), as previously described (Qian *et al*., 2020). The absorbance was measured at 450 nm by using a Synergy H1 Microplate reader (Bio-Tek, USA).

*Caspase-3 activity assay*. Caspase-3 activity was determined using a caspase-3 activity assay kit (ab252897; Abcam, UK). The cells were inoculated on 96-well plates at concentration of $1 \times 10^6$ cells per well. Subsequently, the samples and working solution were prepared and mixed. Fluorescence intensity was measured at 1-h intervals at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Finally, caspase-3 activity was calculated based on the formula provided by the manufacturer.

*Western blotting.* Cells were lysed in radioimmunoprecipitation assay buffer (Beyotechnology, China) and the resulting lysates were mixed with a protease inhibitor cocktail (Beyotechnology). The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Thermo Fischer Scientific, USA). The membranes were blocked with 10% skimmed milk for 1 h at 24°C, after which they were incubated for approximately 24 h at 4°C with primary antibodies against HBV core antigen (1:1,000; Abnova, USA; MAB4775), SIRT1 (1:1,000; Cell Signaling Technology, USA; 2310), and GAPDH (1:1,000; Beyotime; AF1186; internal control). The membranes were then incubated with appropriate secondary antibodies at 24°C for approximately 1 h. Protein bands were detected using enhanced chemiluminescence kits (Invitrogen).

*Animal experiments and hemodynamic replication model.* Twelve BALB/c nude mice (4–6-week-old, male, weight: 18–20 g) were obtained from the animal center of our hospital and housed in animal rooms (10-h light/14-h dark cycle, 22–27°C, and relative

humidity: 40–60%) under sterile conditions with *ad libitum* access to water and standard laboratory chow. HBV plasmid DNA (pHBV; 10 mg) was injected into the mice through the tail vein on day 0, and the mice then received a hydrodynamic injection of pHBV daily for 28 days (Guo *et al*., 2018). The animals were randomly divided into two groups: (1) HBV (n = 6) and (2) HBV + notoginsenoside R1 co-treatment (n = 6) groups. The mice received 50 mg/kg of notoginsenoside R1 by gastric lavage every day for 2 weeks. Once tumors reached 10 mm in diameter, the mice were sacrificed by cervical dislocation and tumor xenografts were collected. All procedures were approved by the Animal Care and Use Committee of Yantai Laiyang Central Hospital (number of permission).

*Staining for HBV core antigen.* Liver tissue samples were first fixed with 4% polyformaldehyde, after which they were embedded and sectioned. After the sections were heated, deparaffinized, rehydrated, and incubated, they were blocked with goat serum for 90 min. Subsequently, they were incubated with a primary mouse anti-HBV core antigen (HBcAg) antibody (dilution: 1:1,000, Abcam; ab8637) overnight at 4°C and followed by secondary antibodies. The sections were counterstained with 3, 3-diaminobenzidine for 4 min, after which they were imaged and analyzed.

*Measurement of HBV antigen and DNA.* HBV surface antigen (HBsAg) and HBV envelope antigen (HBeAg) levels were examined using Abbott Architect i2000SR HBsAg and HBeAg reagent kits (Abbott Diagnostics, USA). HBV DNA was quantified using the Fluorescence quantitative PCR detection kit for HBV DNA (ACON Biotech Co. Ltd, China).

*Statistical analyses.* The data were analyzed using SPSS 22.0 (IBM, USA) and are presented as the mean ± standard deviation. A two-tailed *P* value < 0.05 was set as the cut-off value for significance. All experiments were performed on three independent occasions. Data were analyzed by Student's *t*-test or one-way analysis of variance, with a Bonferroni post hoc test.

## Results

*Notoginsenoside R1 induces the apoptosis of HCC cells*

We first used different concentrations of notoginsenoside R1 to treat HepG2 and MHCC97H cells for 72 h to determine the optimal experimental concentration. We

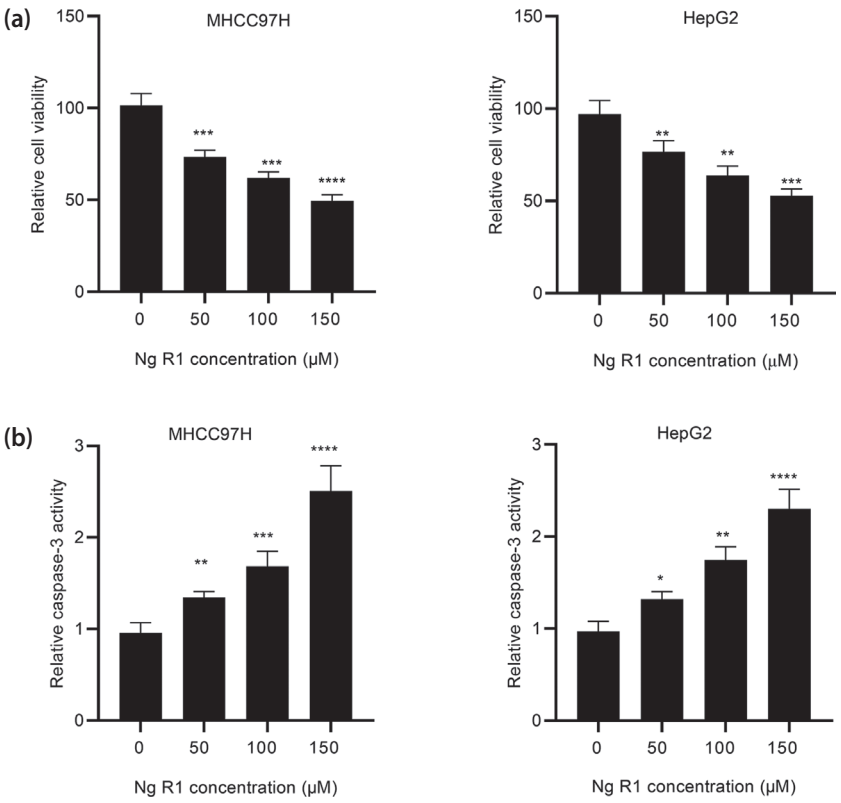


**Fig. 1**

**Notoginsenoside R1 induces the apoptosis of HCC cells**

**(a)** The viability of MHCC97H and HepG2 cells was reduced by notoginsenoside R1 treatment in a dose-dependent manner (0, 50, 100, 150 μM). **(b)** Notoginsenoside R1 increased caspase-3 activity in both MHCC97H and HepG2 cell lines in a dose-dependent manner (0, 50, 100, 150 μM). Ng R1, notoginsenoside R1; NS, non-significant; **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001 versus the control group.

found that the viability of HepG2 and MHCC97H cells was reduced by notoginsenoside R1 treatment in a dose-dependent manner (Fig. 1a). Additionally, notoginsenoside R1 was found to increase caspase-3 activity in both HCC cell lines in a dose-dependent manner (Fig. 1b). Thus, notoginsenoside R1 may exert an apoptotic effect on HCC cells. We chose a notoginsenoside R1 concentration of 150 μM for subsequent experiments.

*Notoginsenoside R1 inhibits HBV replication*

HepG2 and MHCC97H cells (60 mm dishes,1.5 × $10^6$ cells/dish) were treated with notoginsenoside R1 (100 μM) for 72 h. Positive control cells were treated with the SIRT1 inhibitor, selisistat (1 μM). Following treatment, HBV DNA levels were assessed in both cell lines. We found that notoginsenoside R1 and selisistat decreased HBV DNA levels in both cell lines (Fig. 2a). Additionally, pgRNA and *preC* RNA levels in the cells were assessed to explore the mechanism underlying the suppression of HBV replication by notoginsenoside R1 and selisistat. We found that following notoginsenoside R1 treatment, pgRNA, preC, HBsAg, and HBeAg levels were reduced in both cell lines (Fig. 2b-f). Additionally, the levels of HBcAg protein in the cells were examined by western blotting. HBcAg protein expression levels were decreased by notoginsenoside R1 and selisistat treatment in both cell lines (Fig. 2f). Thus, these data suggested that notoginsenoside R1 suppressed

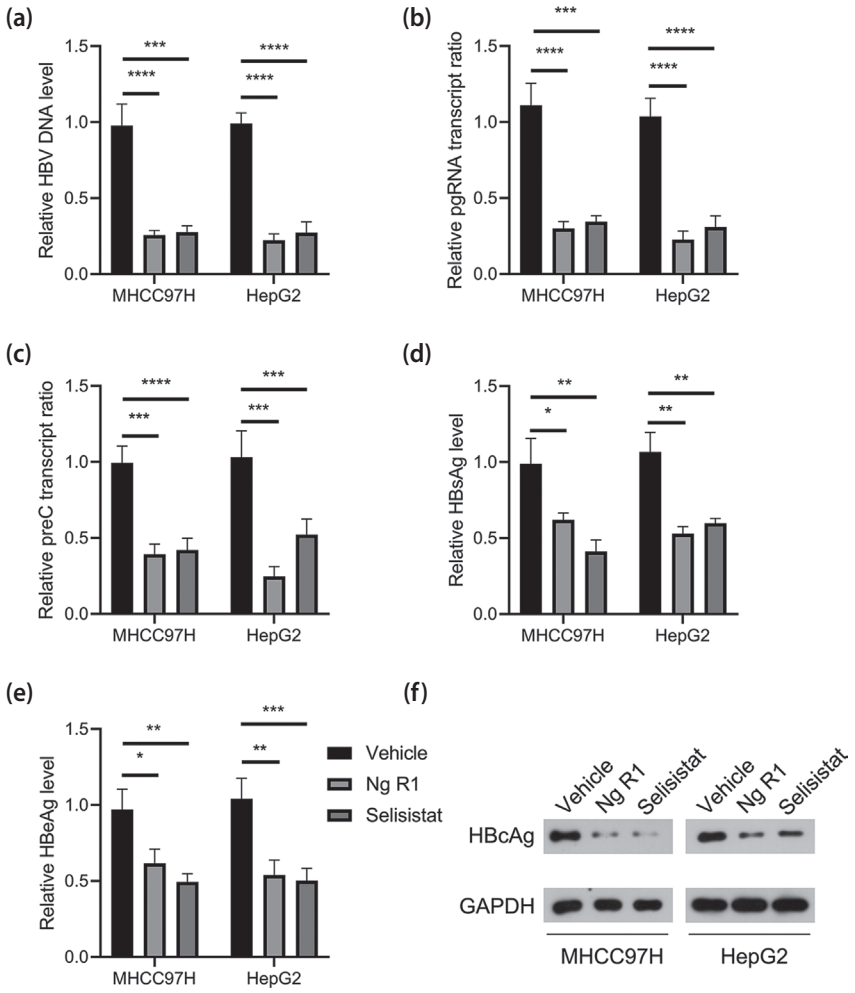


**Fig. 2**

**Notoginsenoside R1 and selisistat decrease the replication of HBV**

Notoginsenoside R1 (100 μM) and selisistat (1 μM) decreased **(a)** HBV DNA levels, **(b)** HBV pregenomic RNA levels, **(c)** HBV *preC* RNA levels, (d) HBV HBsAg levels, and **(e)** relative HBV HBeAg levels in both MHCC97H and HepG2 cell lines. **(f)** Western blot showing HBcAg expression levels decreased by notoginsenoside R1 (100 μM) and selisistat (1 μM) treatment in both MHCC97H and HepG2 cell lines. Ng R1, notoginsenoside R1. NS, non-significant; **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001 versus the control group.

HBV replication by interacting with its core promoter region and that suppressing SIRT1 activity decreased the replication of HBV.

*Notoginsenoside R1 inhibits HBV replication by downregulating SIRT1 activity*

Following notoginsenoside R1 treatment, *SIRT1* mRNA expression levels were downregulated (Fig. 3a). The protein levels of SIRT1 were measured by western blotting and were found to be significantly reduced by notoginsenoside R1 treatment (Fig. 3b). To validate the finding that the downregulation of SIRT1 expression leads to an antiviral effect, SIRT1 expression was downregulated in the two cell lines by transfection with si-*SIRT1* (Fig. 3c,d). The cells were then treated with notoginsenoside R1 and HBV DNA levels were measured. The results indicated that silencing SIRT1 inhibited HBV replication. (Fig. 3e). Thus, notoginsenoside R1 exerts its antiviral effect on HCC cells primarily by decreasing SIRT1 activity.

*Notoginsenoside R1 inhibits HBV replication in vivo*

A mouse model of HBV replication was established, as previously described, by injecting each mouse with 1.3-mer HBV genomic DNA (10 μg per mouse). Following injection, notoginsenoside R1 was administered by gastric lavage for 2 weeks. Serum HBV DNA levels were markedly lower in notoginsenoside-R1-treated mice than in control mice (Fig. 4a). Moreover, circulating HBsAg and HBeAg levels were measured, and found to be lower in the treatment group than in the control group (Fig. 4b,c). These data indicate that notoginsenoside R1 suppresses HBV replication *in vivo*.

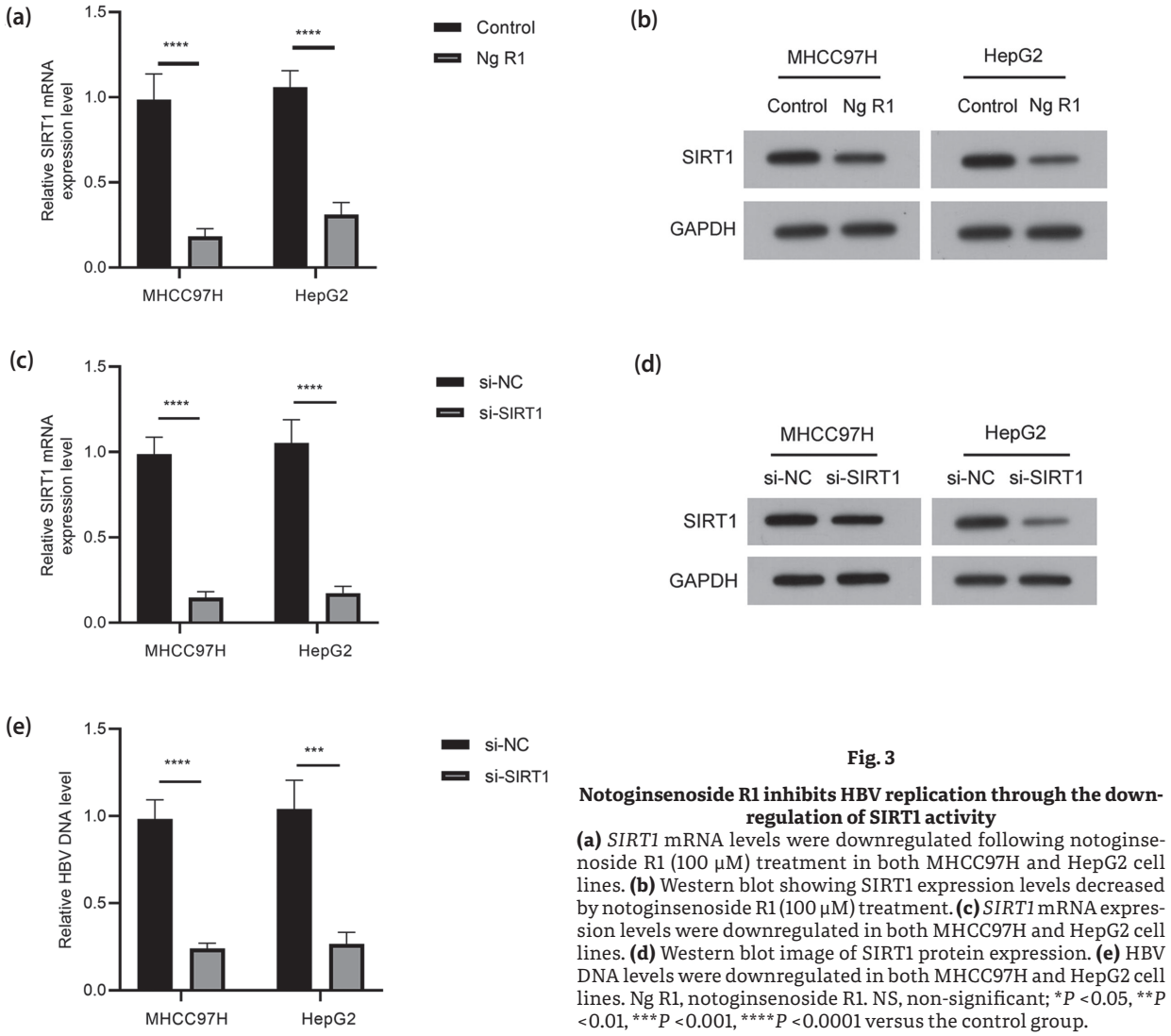


**Fig. 3**

**Notoginsenoside R1 inhibits HBV replication through the downregulation of SIRT1 activity**

**(a)** *SIRT1* mRNA levels were downregulated following notoginsenoside R1 (100 μM) treatment in both MHCC97H and HepG2 cell lines. **(b)** Western blot showing SIRT1 expression levels decreased by notoginsenoside R1 (100 μM) treatment. **(c)** *SIRT1* mRNA expression levels were downregulated in both MHCC97H and HepG2 cell lines. **(d)** Western blot image of SIRT1 protein expression. **(e)** HBV DNA levels were downregulated in both MHCC97H and HepG2 cell lines. Ng R1, notoginsenoside R1. NS, non-significant; **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001 versus the control group.

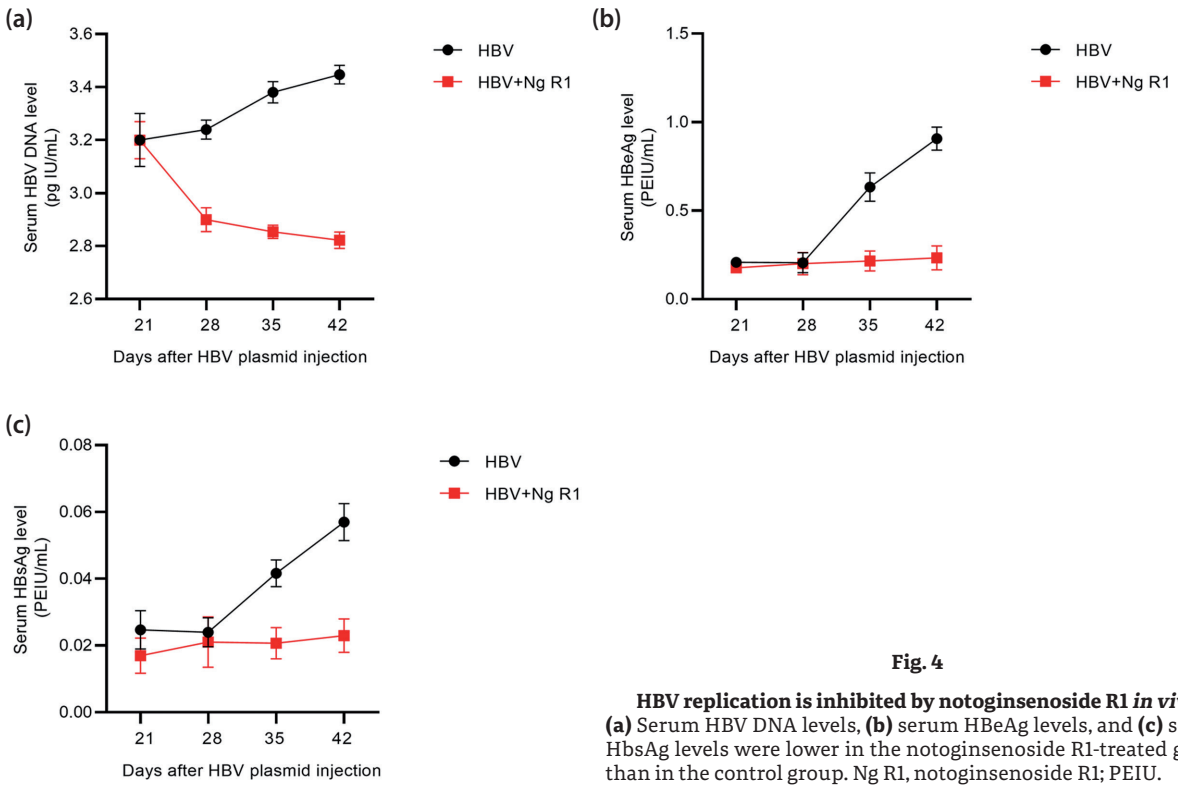


**Fig. 4**

**HBV replication is inhibited by notoginsenoside R1 *in vivo***
**(a)** Serum HBV DNA levels, **(b)** serum HBeAg levels, and **(c)** serum HbsAg levels were lower in the notoginsenoside R1-treated group than in the control group. Ng R1, notoginsenoside R1; PEIU.

*Notoginsenoside R1 suppresses HBV replication via SIRT1*

The results of HBcAg staining confirmed that notoginsenoside R1 decreases HBV replication (Fig. 6a). Western blotting analysis of mouse livers showed that HBcAg expression levels were decreased by notoginsenoside R1 (Fig. 8b). Moreover, notoginsenoside R1 significantly decreased HBV DNA levels in the mouse livers (Fig. 5c). Notably, *SIRT1* mRNA expression was downregulated in the livers of the mice in the notoginsenoside R1 treatment group (Fig. 5d). Therefore, notoginsenoside R1 suppressed HBV replication *in vivo*.

## Discussion

The various pharmacological effects of notoginsenoside R1 have attracted considerable attention; however, its effects on HBV have not yet been studied. To the best of our knowledge, the present study is the first to report that notoginsenoside R1 significantly suppresses HBV replication, both *in vitro* and *in vivo*. Although notoginsenoside R1 has not been shown to have inhibitory effects on the replication of other viruses, other ginsenosides have been shown to have suppressive effects on virus replication. For instance, ginsenoside Rg3 inhibits HBV replication by promoting the degradation of TRAF6/TAK1 and inhibiting JNK/AP-1 signaling (Kang *et al.*, 2013). Additionally, ginsenoside Rg3 exhibits a suppressive effect on the propagation of HBV by regulating mitophagy (Kim *et al.*, 2017). Furthermore, ginsenoside R1 exerts a protective effect on alcoholic hepatitis by decreasing NF-κB activity (Gao *et al.*, 2015). Notably, ginsenoside R1 reduces the apoptosis and inflammation of hepatocytes during ischemia-reperfusion injury (Chen *et al.*, 2008). Based on these findings, we hypothesized that notoginsenoside R1 may be a novel agent to treat HBV infection.

Furthermore, it was discovered that notoginsenoside R1 exerted an antiviral effect primarily through the inhibition of SIRT1 activity. Although SIRT1 exhibits an inhibitory effect against certain viruses, many studies have revealed that it may promote the replication of HBV. Deng *et al.* discovered that SIRT1 recruits HBV X protein to facilitate HBV transcription (Deng *et al.*, 2017). Additionally, it regulates the expression of the transcription factor, AP-1, to increase HBV replication (Ren *et al.*, 2014). Another study confirmed that SIRT1 enhances HBV transcriptional activity by interacting with PGC-1α and PPARα (Shi *et al.*, 2016). Furthermore, HBV replication is suppressed by the SIRT1 inhibitor, nicotinamide (Li *et al.*, 2016). In the present study, we showed that inhibiting

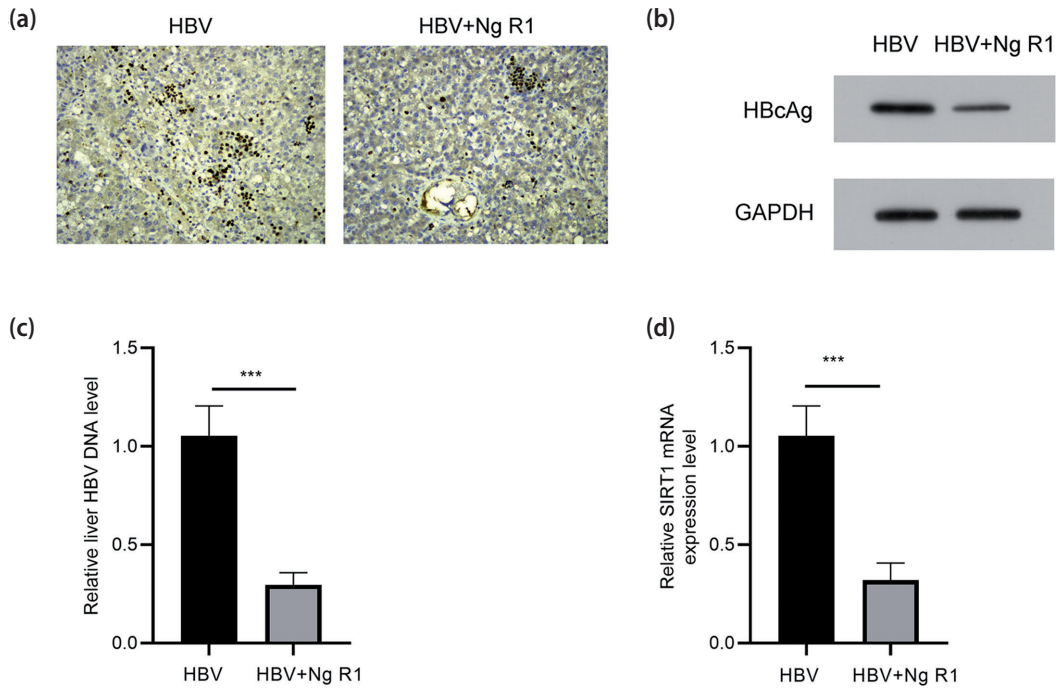


**Fig. 5**

**Notoginsenoside R1 suppresses HBV replication via SIRT1**

**(a)** HBcAg staining showed that notoginsenoside R1 decreased HBV replication. **(b)** Western blot showing HBcAg expression levels decreased by notoginsenoside R1. **(c)** HBV DNA levels were decreased by notoginsenoside R1. **(d)** *SIRT1* mRNA expression levels were downregulated in the notoginsenoside-R1-treatment group. Ng R1, notoginsenoside R1. NS, non-significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus the control group.

SIRT1 activity, either through the knockdown of SIRT1 or by using SIRT1 inhibitors, suppressed the replication of HBV. However, inhibiting SIRT1 activity may result in some complications. It has been reported that hepatic lipid metabolism is regulated by SIRT1 via the activation of the AMPK signaling pathway (Hou *et al.*, 2008). Additionally, it has been found that the depletion of SIRT1 may lead to the accumulation of fatty acids in hepatocytes, resulting in hepatic inflammation (Purushotham *et al.*, 2009). Moreover, SIRT1 activates FGF21 to control energy metabolism in the liver of rats (Li *et al.*, 2014). Thus, there is a possibility that decreasing SIRT1 activity may result in hepatic steatosis.

Our study has some limitations that should be noted. First, additional clinical data are required to examine the expression levels of SIRT1 in healthy individuals and patients with HBV infection. Second, clinical trials are necessary to confirm the antiviral effects of notoginsenoside R1. Third, future studies should continue to investigate the potential side effects of notoginsenoside R1 in clinical practice.

In conclusion, we found that notoginsenoside R1 exerts an inhibitory effect on HBV infection, primarily by downregulating SIRT1 activity. This discovery opens a new direction for the development of treatments for HBV infection.

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