Sodium Selenite Suppresses Hepatitis B Virus Transcription and Replication in Human Hepatoma Cell Lines

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Hepatitis B virus (HBV) infection is one of the most serious and prevalent health problems worldwide. Current anti-HBV medications have a number of drawbacks, such as adverse effects and drug resistance; thus, novel potential anti-HBV reagents are needed. Selenium (Se) has been shown to be involved in both human immunodeficiency virus and hepatitis C virus infections, but its role in HBV infection remains unclear. To address this, sodium selenite (Na_2SeO_3) was applied to three HBV cell models: HepG2.2.15 cells, and HuH-7 cells transfected with either 1.1 or 1.3× HBV plasmids. Cytotoxicity of Na_2SeO_3 was examined by Cell Counting Kit-8. Levels of viral antigen expression, transcripts, and encapsidated viral DNA were measured by enzyme-linked immunosorbent assay, northern blot, and Southern blot, respectively. There was no obvious cytotoxicity in either HepG2.2.15 or HuH-7 cells with $<2.5\,\mu M$ Na_2SeO_3. Below this concentration, Na_2SeO_3 suppressed HBsAg and HBeAg production, HBV transcript level, and amount of genomic DNA in all three tested models, and suppression level was enhanced in line with increases in Na_2SeO_3 concentration or treatment time. Moreover, the inhibitory effect of Na_2SeO_3 on HBV replication can be further enhanced by combined treatment with lamivudine, entecavir, or adefovir. Thus, the present study clearly proves that Na_2SeO_3 suppresses HBV protein expression, transcription, and genome replication in hepatoma cell models in a dose- and time-dependent manner. ***J. Med. Virol.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B virus; selenium; sodium selenite; suppression

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem throughout the world, especially in East Asia and Africa. An estimated two billion people, roughly 30% of the world's population, have been infected with HBV, and over 350 million patients have chronic hepatitis B (CHB) [Liaw and Chu, 2009]. Each year, about 786,000 deaths are caused by HBV infection or its associated liver diseases [Lozano et al., 2012], including hepatic decompensation, cirrhosis, and hepatocellular carcinoma; HBV-infected individuals are at higher risk of all these HBV related diseases [Lee et al., 2013]. Although efficient prophylactic vaccines have been developed for prevention of HBV infection, there is still no cure for existing infection. The current clinical applied therapeutic agents for CHB are mainly immunomodulators and nucleoside/nucleotide analogues, which can be used either separately or in combination [Yuen and Lai, 2001; Papatheodoridis et al., 2008; Scaglione and Lok, 2012; Trepo et al., 2014]. Interferon alpha (IFN-α) is the best-known immunodulator, and controls HBV infection by stimulating the cellular antiviral cytokine expression to inhibit viral replication and by enhancing the host

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immune responses to eliminate the HBV-infected hepatocytes [Yuen and Lai, 2001]. However, its poor response against CHB, high commercial cost, and various adverse effects (AEs) limit the wide clinical application of this drug.

Multiple nucleoside (lamivudine, entecavir, and telbivudine) and nucleotide analogues (adefovir and tenofovir) have been approved for clinical application [Liaw and Chu, 2009]. These oral antiviral agents mainly target HBV polymerase and reverse transcriptase by rapidly and potently inhibiting their activities. Although nucleoside/nucleotide analogues have fewer AEs, they fail to eradicate HBV covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes, which results in viral relapse after stopping the medication [Yuen and Lai, 2001; Trepo et al., 2014]. In addition, the development of drug resistance during therapy also limits the effective application of nucleoside/nucleotide analogues. Some of the most recent drugs, such as entecavir and tenofovir, have been observed to induce only a very low level of drug resistance, but long-term application may still induce expansion of HBV strains with mutations resisting these drugs [Trepo et al., 2014]. Thus, developing novel effective therapeutic approaches and agents is a necessary step against HBV infection.

Selenium (Se) is an essential trace mineral for animals and humans. In addition to antioxidant activity, Se has also shown anti-inflammatory and anticancer properties [Rayman, 2000]. Se functions in either its inorganic forms, such as sodium selenite (Na_2SeO_3) and sodium selenate, or its organic forms, such as the amino acids selenocysteine (Sec) and selenomethionine (SeMet), and multiple selenomolecles as intermediary metabolites [Papp et al., 2007]. Upon absorption, the inorganic salts in their oxidized forms [selenite (Se^{4+}) or selenate (Se^{6+})] can be reduced to selenide (Se^{2-}) by using reducing equivalents from reduced glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH) [Zeng and Combs, 2008]. As a storage mechanism, SeMet can replace normal methionine in protein synthesis, and then be released reversibly by the normal metabolic process as necessary [Schrauzer, 2000]. Sec is probably the most abundant biologically active form of selenium in vivo, and can be specially incorporated into selenoproteins as the 21st amino acid encoded by the UGA codon [Metanis and Hilvert, 2014]. Importantly, Sec might play some important biological roles in vivo, as most of the 25 identified human selenoproteins have shown enzymatic redox function with catalytic or antioxidant activities conferred by Sec [Kryukov et al., 2003; Papp et al., 2007].

Besides the biological functions described above, Se is also involved in the occurrence, virulence, and disease progression of some viral infections [Rayman, 2000; Moghadaszadeh and Beggs, 2006]. For some RNA viruses, including coxsackievirus B3 (CVB3/0) (cause of Keshan disease), human immunodeficiency virus (HIV), influenza A virus, SARS coronavirus, and Ebola virus, the absence of Se causes accumulation of mutations in their genome, leads to changes in the virulence-associated genetic structures [Beck et al., 2003; Harthill, 2011]. Viral glutathione peroxidase (vGPx), a selenoprotein with antioxidant activity, has been found to be encoded by some RNA virus such as HIV and hepatitis C virus [Zhang et al., 1999]. In virus-infected cells, vGPx inhibits ROS-induced apoptosis, a host response against infection, and further promotes viral replication [Papp et al., 2007].

To date, only a few reports have described the possible relevance of Se to HBV infection [Yu et al., 1997; Khan et al., 2012], but direct evidence is still lacking, and the underlying mechanism remains to be elucidated. In the present study, hepatoma cell HepG2.2.15 and HuH-7 transfected with two different HBV plasmids were utilized in order to investigate the effect of Na_2SeO_3 on HBV. The data clearly clarify that application of Na_2SeO_3 inhibits HBsAg/HBeAg expression, HBV transcription, and genome replication in the examined models. This is the first report with direct evidence confirming the suppressive effect of Se on HBV replication.

MATERIALS AND METHODS

Cells and Plasmids

The human hepatoma cell lines HepG2.2.15 and HuH-7 were obtained from the China Center for Type Culture Collection (CCTCC), and maintained in Dulbecco's modified eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 mg/ml penicillin, and 100 μg/ml streptomycin (all from Gibco Life Technologies) at 37°C in a 5% CO_2 incubator. HepG2.2.15 is derived from HepG2 cells by being stably transfected with a construct containing two head-to-tail dimers of the HBV genome (GenBank accession: U95551.1), so 200 μg/ml G418 was also added to maintain the cell line.

Plasmid pCH-9/3091 (1.1×HBV) is an HBV construct containing 1.1 copies of the HBV genome (subtype ayw) driven by the human cytomegalovirus (HCMV) immediate early I protein (IE1) promoter [Nassal, 1992] (kind gift from Dr Michael Nassal), and p1.3HBV (1.3×HBV) is another HBV construct generated by inserting 1.3 copies of the HBV genome (subtype adw), starting from the enhancer-I-X promoter region, into pGEM-3Z [Doitsh and Shaul, 2003] (kind gift from Dr. Yosef Shaul).

Transfection and Treatment

To study viral replication, plasmid pCH-9/3091 and 1.3×HBV were transfected into 3×10^6 HuH-7 cells in 6 cm plates using Lipofectamine™ 2000 (Invitrogen, Waltham, MA) according to the manufacturer's instructions. After HepG2.2.15 was cultured for 24 hr

or HuH-7 was transfected for 24 hr, then the medium was replaced by fresh medium with Na_2SeO_3 or 30 nM entecavir (ETV) (both Sigma Aldrich, St. Louius, MO), which had been dissolved in 1× sterile phosphate-buffered saline (PBS). The treatment starting day was defined as Day 0, and samples were collected at the indicated time points.

Cytotoxicity Assay

HepG2.2.15 and HuH-7 cells (1×10^4 cells/well) were cultured in 96-well plates, and Na_2SeO_3 at the indicated concentrations was added to the culture 24 h later. The cells were harvested at the indicated time points, and assayed by colorimetry (Cell Counting Kit-8; Dojindo Laboratories).

Enzyme-Linked Immunosorbent Assay of HBV Antigens

To quantify HBsAg and HBeAg production, the supernatants of the cells with the indicated treatment were collected, and tested for the presence of HBsAg and HBeAg using a commercial enzyme-linked immunosorbent assay (ELISA) kit (KEHUA Bio-engineering). The results were normalized to the mock-treatment (PBS) control sample.

Northern Blot Assay for Viral RNA

Cells were washed twice with PBS and homogenized in TRIzol (Invitrogen). Total RNA was isolated according to the manufacturer's protocol. Potential DNA contamination in the extraction was eliminated with recombinant RNase-free DNase I (TaKaRa). Samples were incubated at 65°C for 5 min for denaturation, and then 20 μg RNA was electrophoresed for 3 hr at 100 V in 1.2% (w/v) agarose gel in the presence of formaldehyde. As described previously [Tian et al., 2013], the resolved RNA was then blotted onto a positively charged nylon membrane, and hybridized with a ^{32}P-labeled random-primed probe specific for the HBV genome. The visualized image was obtained using Cyclone Plus Storage Phosphor System (PerkinElmer, Waltham, MA), and the cell level in the northern blot assay were normalized to 28S/18S RNA.

Southern Blot Assay for Encapsidated HBV DNA

Intracellular viral core DNA was isolated using the method described previously [Feng et al., 2013]. Briefly, cells were lysed in either 1 ml (HepG2.2.15, 10^7 cells per 10 cm plate,) or 0.6 ml (HuH-7, 3×10^6 cells per 6 cm plate) lysis buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.5% NP-40). After removing the cell debris by centrifugation, the supernatants were treated with 60 U DNase I (TaKaRa) and 5 μl RNase A (TaKaRa), and then incubated at 37°C for 2 hr in the presence of 8 mM $MgCl_2$ to completely digest the non-encapsidated DNA and RNA. Core particles were degraded with 200 mM proteinase K (TIANGEN), and encapsidated DNA was extracted with phenol chloroform and precipitated by ethanol. Following separation in a 1% agarose gel (w/v), the isolated encapsidated HBV DNA was blotted onto a positively charged nylon membrane (Millipore) and hybridized with the same probe used for the northern blot assay. Finally, the signals were visualized using Cyclone Plus Storage Phosphor System (Perkin Elmer). The cell level in the Southern blot assay was normalized to the β-actin protein level.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The quantitative real-time polymerase chain reaction was performed with the SYBR Premix Ex Taq II (TaKaRa) using a LightCycler 96 Real-Time PCR system (Roche, Penzberg, Germany). The HBV genome sequence (GeneBank Accession no. DQ219811) was amplified with primer 5′-ACC AAT CGC CAG TCA GGA AG-3′ and 5′-ACC AGC AGG GAA ATA CAG GC-3′; and β-actin (GeneBank Accession no. NM_001101), serving as the internal reference, was amplified with primer 5′-CAT GTA CGT TGC TAT CCA GGC-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT-3′. The cycling program was run at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec.

Statistical Analysis

All results for the toxicity assay and ELISA are shown as mean ± standard error from at least three independent experiments. Statistical differences were analyzed using unpaired two-tailed Student's t tests. Differences were considered to be statistically significant at $P < 0.05$. Results of Southern or northern blot assays are representative images selected from at least three independent experiments.

RESULTS

Cytotoxicity of Na_2SeO_3 to HepG2.2.15 and HuH-7 Cells

To eliminate the possibility that any observed influences of Na_2SeO_3 on HBV replication were caused by cytotoxicity, the influence of Na_2SeO_3 on HuH-7 and HepG2.2.15 proliferation was examined using CCK-8 assay. No significant toxicity of Na_2SeO_3 on HuH-7 cells was observed at concentrations 2.5 μM, whereas Na_2SeO_3 treatment beyond this concentration displayed inhibition of HuH-7 growth in a dose-dependent manner (Fig. 1A). HepG2.2.15 cells showed greater tolerance against Na_2SeO_3 treatment. Treatment with 10 μM Na_2SeO_3 for three days and 5 μM Na_2SeO_3 for five days did not change the growth of HepG2.2.15 compared with the mock-treated control. Beyond this concentration, the cell numbers of HepG2.2.15 decreased gradually, indicating cytotoxicity of Na_2SeO_3 (Fig. 1B). For both cell types, no significant differences were observed during

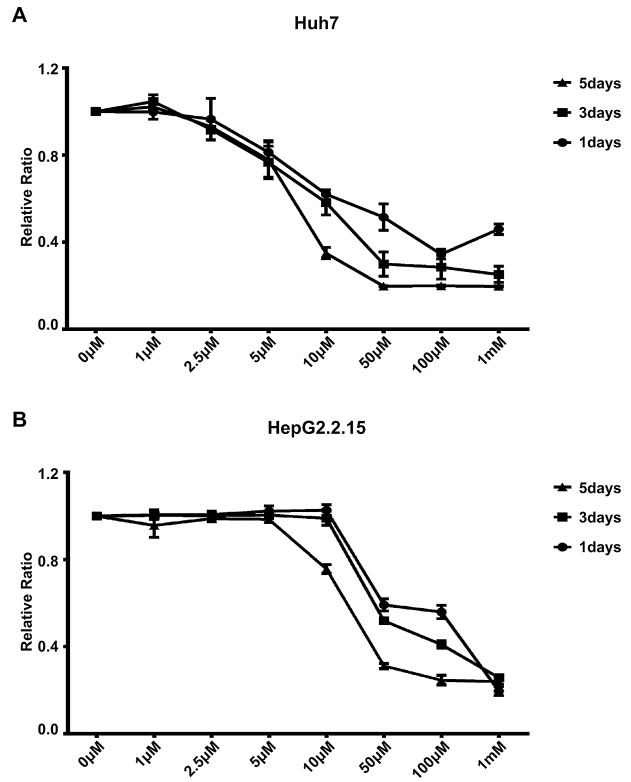

Fig. 1. Cytotoxicity of Na_2SeO_3. HuH-7 (**A**) or HepG2.2.15 (**B**) cells were treated with Na_2SeO_3, and assayed for cell viability at the indicated time points. The results were normalized to the mock-treated control.

the Na_2SeO_3 treatment period at or below the safe concentration of 2.5 μM for HuH-7 and 5 μM for HepG2.2.15. However, the numbers of viable cells were obviously decreased during the treatment period beyond the safe concentration (Fig. 1A and B). For HuH-7, the decline in cell growth at one, three, and five days was relatively consistent (Fig. 1A), but HepG2.2.15 treated for five days showed a more dramatic decrease in cell growth compared with Days one and three (Fig. 1B).

Hence, 2.5 μM, which showed no significant cytotoxicity for either cell type, was set as the maximum concentration of Na_2SeO_3 treatment for subsequent experiments.

Inhibition of HBsAg and HBeAg Production by Na_2SeO_3

To study the effect of Na_2SeO_3 on HBV viral protein production, HepG2.2.15 cells or HBV plasmid-transfected HuH-7 cells were treated with Na_2SeO_3. Four concentrations, 0.5, 1.5, 2.0, and 2.5 μM, of Na_2SeO_3 were investigated for cytotoxicity (Fig. 1). PBS and 30 nM ETV were used as controls. At three days post-treatment (HuH-7) or post-culture (HepG2.2.15), supernatants of the cultures were collected, and HBsAg and HBeAg expression were measured by ELISA. For HepG2.2.15, 0.5 μM Na_2SeO_3 was enough to cause a significant reduction in HBsAg and HBeAg expression compared with the mock-treated control, and Na_2SeO_3 at the examined concentrations inhibited HBV antigen production in HepG2.2.15 in a dose-dependent manner (Fig. 2A and B).

In HuH-7 cells transfected with 1.1× HBV plasmid, 0.5 μM Na_2SeO_3 failed to decrease HBsAg and HBeAg levels. A dose-dependent suppression was observed, starting from 1.5 μM Na_2SeO_3 (Fig. 2C and D). Viral antigen in 1.3× HBV plasmid-transfected HuH-7 cells showed even stronger resistance to Na_2SeO_3. HBsAg and HBeAg levels started to decline with 2 μM Na_2SeO_3, and the decline was further enhanced by 2.5 μM Na_2SeO_3 treatment (Fig. 2E and F).

Next, a fixed concentration (2.5 μM) of Na_2SeO_3 was used to assess viral antigens expression over a time course. For HepG2.2.15 cells, 2.5 μM Na_2SeO_3 showed significant inhibition of HBV antigen expression from Day 2, and these repressive effects increased with time (Fig. 3A and B). On the final assessment day (Day 4), 2.5 μM Na_2SeO_3 treatment had caused a reduction of $62.81 \pm 2.59\%$ and $54.69 \pm 7.88\%$ in production of HBsAg and HBeAg, respectively. A similar time-dependent suppression enhancement was also observed in HBV plasmid-transfected HuH-7 cells, with the maximum decrease in viral antigen levels also occurring on Day 4. Compared with the mock-treated control, 2.5 μM Na_2SeO_3 treatment in HuH-7 cells transfected with 1.1 or 1.3× HBV plasmid produced a drop in HBsAg level to $41.16 \pm 10.55\%$ and $42.14 \pm 10.15\%$ (Fig. 3C and E), respectively, and a drop in HBeAg level to $29.73 \pm 8.33\%$ and $32.32 \pm 9.03\%$, respectively (Fig. 3D and F). There was no obvious decrease in 1.3× HBV plasmid-transfected HuH-7 cells on Day 1, but at this time point, HBeAg was already significantly suppressed in 1.1× HBV plasmid-transfected HuH-7 cells (Fig. 3D).

Notably, ETV treatment did not affect viral antigen production in any of the models (Figs. 2 and 3).

Suppression of HBV Transcription by Na_2SeO_3

Because HBsAg and HBeAg are products of preS/S mRNAs and precore mRNA, levels of various HBV mRNAs were further analyzed in HepG2.2.15 and 1.1/1.3× HBV plasmid-transfected HuH-7 cells in the presence of Na_2SeO_3. Four concentrations, 0.5, 1.5, 2.0, and 2.5 μM, of Na_2SeO_3 were used, and the concentration of 2.5 μM was further analyzed at different time points. The HBV RNAs in all the examined models were decreased by Na_2SeO_3 treatment, but with slight differences.

In HepG2.2.15 cells, 0.5 μM Na_2SeO_3 had no obvious effect on viral transcription, but 1.5 μM dramatically decreased HBV RNA level, which was further decreased with 2.0 μM Na_2SeO_3 treatment (Fig. 4A). When the concentration was fixed at 2.5 μM, progressive repression by Na_2SeO_3 of HBV

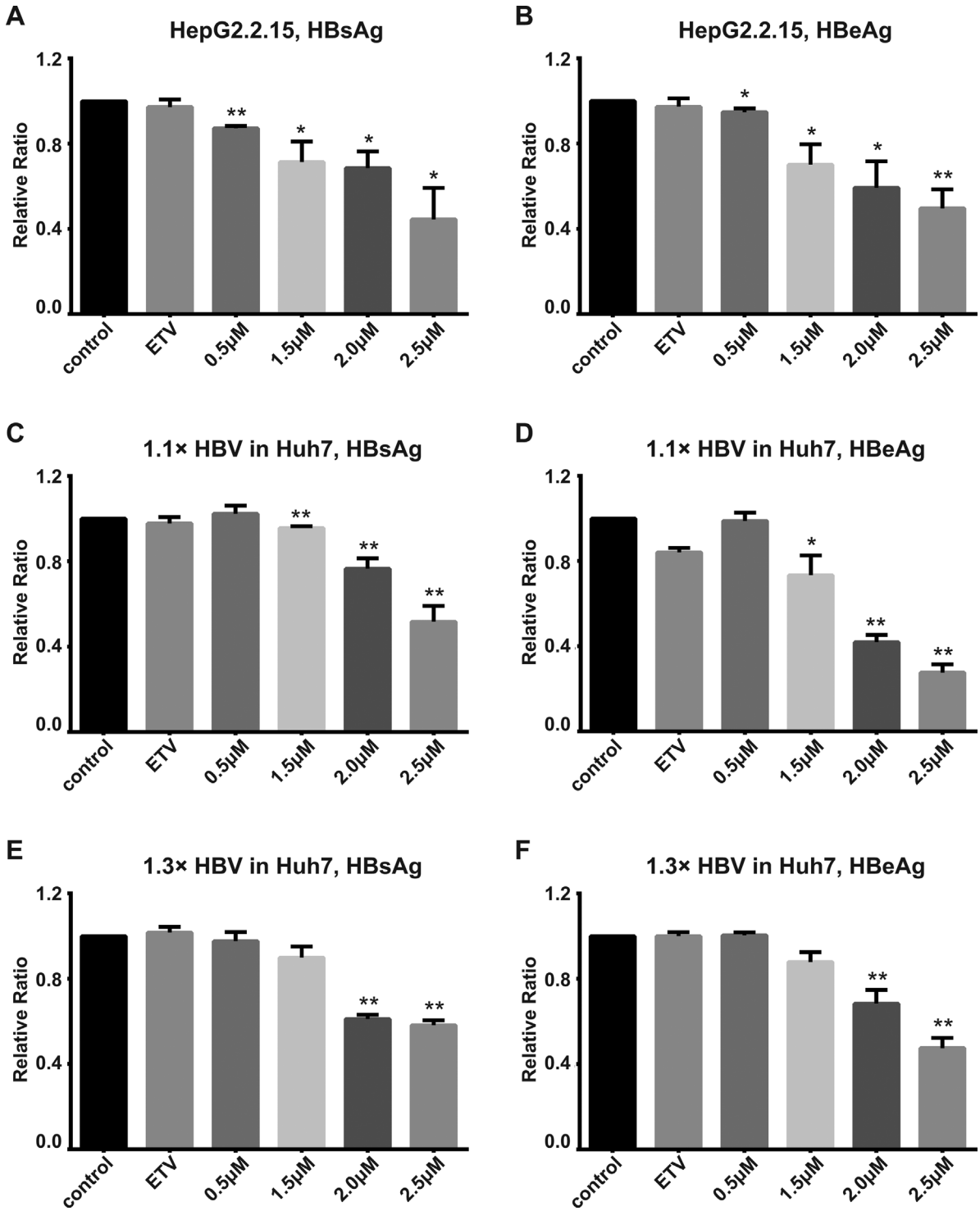

Fig. 2. Dose-dependent suppression of HBsAg/HBeAg expression by Na_2SeO_3. HepG2.2.15 and 1.1× or 1.3× HBV-transfected HuH-7 cells were treated with Na_2SeO_3 at the indicated concentrations or with 30 nM ETV. On Day 3, culture supernatants were collected and assayed for HBsAg and HBeAg concentration by ELISA. All data were normalized to the mock-treated control. $^{*}P < 0.05$; $^{**}P < 0.01$.

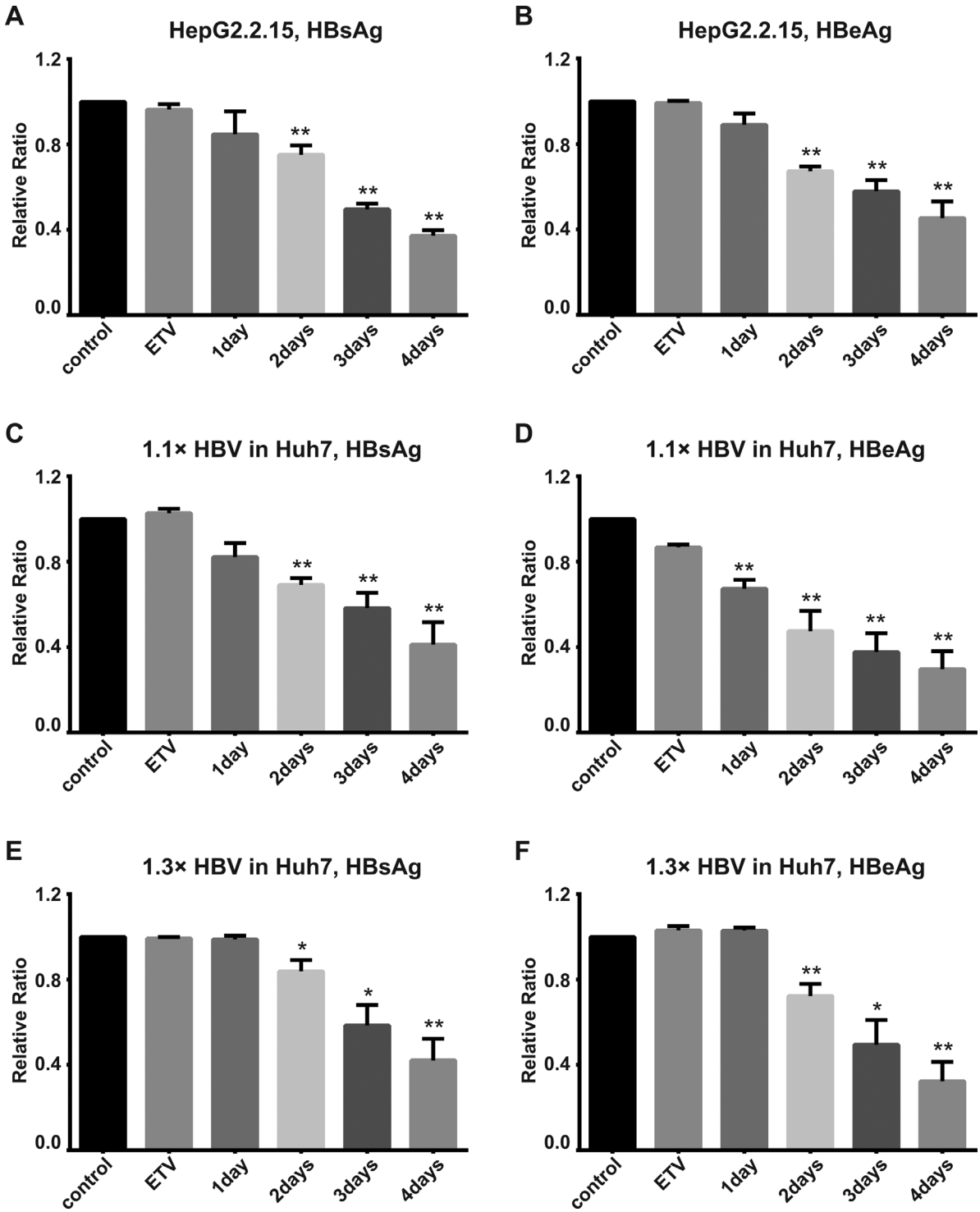

Fig. 3. Time-dependent suppression of HBsAg/HBeAg expression by Na_2SeO_3. HepG2.2.15 and 1.1×or 1.3× HBV-transfected HuH-7 cells were treated with 2.5 μM Na_2SeO_3 or 30 nM ETV. Supernatants of the Na_2SeO_3-treated cultures were collected daily for four days, and ETV-treated supernatant was harvested on Day 4. HBsAg and HBeAg concentrations were measured by ELISA. All data were normalized to the mock-treated control. $^{*}P < 0.05$; $^{**}P < 0.01$.

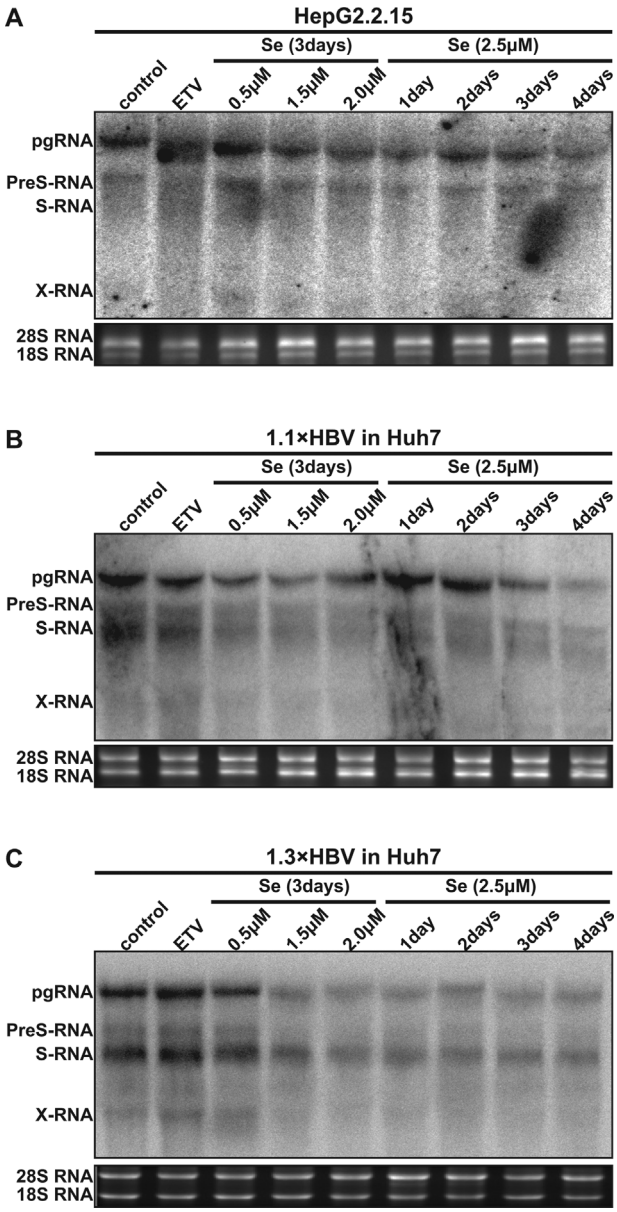

Fig. 4. Suppression of HBV transcription by Na_2SeO_3. HepG2.2.15 and 1.1 or 1.3×HBV-transfected HuH-7 were treated with Na_2SeO_3. Partial samples were treated with various concentrations of Na_2SeO_3 and harvested on Day 3, and some samples were treated with a fixed concentration of 2.5 μM Na_2SeO_3, but harvested at different time points. Mock-treated control and 30 nM ETV-treated cells were collected on Day 4. HBV RNA levels were determined by northern blot assay.

RNA level in HepG2.2.15 cells occurred in line with the length of treatment, starting from Day 1 (Fig. 4A). Moreover, the viral transcription level in HepG2.2.15 was quantified using qPCR, and the host gene β-actin was chosen as the internal reference. The result of qPCR (Fig. S1) was consistent with northern blot assay (Fig. 4A). Viral mRNA was inhibited by Na_2SeO_3 treatment in dose-dependent and time-dependent manners, which was reduced to $49.28 \pm 3.82\%$ in the presence of 2.5 μM Na_2SeO_3 for four days. In HuH-7 cells transfected with 1.1×HBV plasmids, there was a dose-dependent suppression of viral mRNA by Na_2SeO_3 (Fig. 4B). In addition, perceptible repression of HBV transcription was observed from Day 2 of 2.5 μM Na_2SeO_3 treatment, which also occurred in a time-dependent manner (Fig. 4B). Na_2SeO_3-induced suppression of HBV transcripts also occurred in HuH-7 cells transfected with 1.3×HBV plasmids. As shown in Figure 4C, suppression was evident with as little as 0.5 μM Na_2SeO_3, and progressed in a dose-dependent manner. Na_2SeO_3 treatment at 2.5 μM caused a dramatic suppression of HBV RNAs from Day 1; however, the time-dependent suppression effect was not as clear as in the other two models. This was probably because the RNA amount had already dropped to a very low level, and therefore the effects at the subsequent examined time points were less obvious (Fig. 4C). In all models, 30 nM ETV treatment produced no effect on HBV transcription levels, because it specifically inhibits protein-priming activity [Jones et al., 2013].

Intracellular viral core protein was also determined using western blot assay in all three models, with the host protein β-actin as the internal reference. Core protein level was decreased gradually following Na_2SeO_3 treatment in a dose-dependent manner, whereas β-actin level remained unchanged (Fig. S2). But 30 nM ETV treatment could not affect core expression in all models (Fig. S2). The result indicated that Na_2SeO_3 influence specifically HBV transcription and expression.

Suppression of HBV DNA Replication by Na_2SeO_3

The fact that Na_2SeO_3 repressed HBV protein synthesis and transcription in a dose-dependent and time-dependent manner prompted us to investigate whether it also influenced HBV DNA levels. To address this issue, the encapsidated HBV DNA was extracted from the cells treated with Na_2SeO_3 or ETV, and analyzed by Southern blot assay. In all models, the viral nucleic acids were barely detectable after 30 nM ETV treatment for three days (Fig. 5A, C, and E) or four days (Fig. 5B, D, and F). Considering that ETV hardly changed the level of viral proteins or RNAs, the dramatic inhibition of HBV DNA was consistent with its specific suppression of HBV replication. A previous study also reported that 30 nM ETV was sufficient to reduce HBV DNA level by 90% [Bader and Korba, 2010], which is in agreement with this result.

In general, Na_2SeO_3 treatment displayed a clear dose-dependent and time-dependent suppression of HBV DNA level in all models (Fig. 5), although some slight differences still existed between the different models. Treatment with Na_2SeO_3 resulted in a very obvious repression of HBV DNA in HepG2.2.15 cells. Clear DNA decline occurred with 0.5 μM Na_2SeO_3

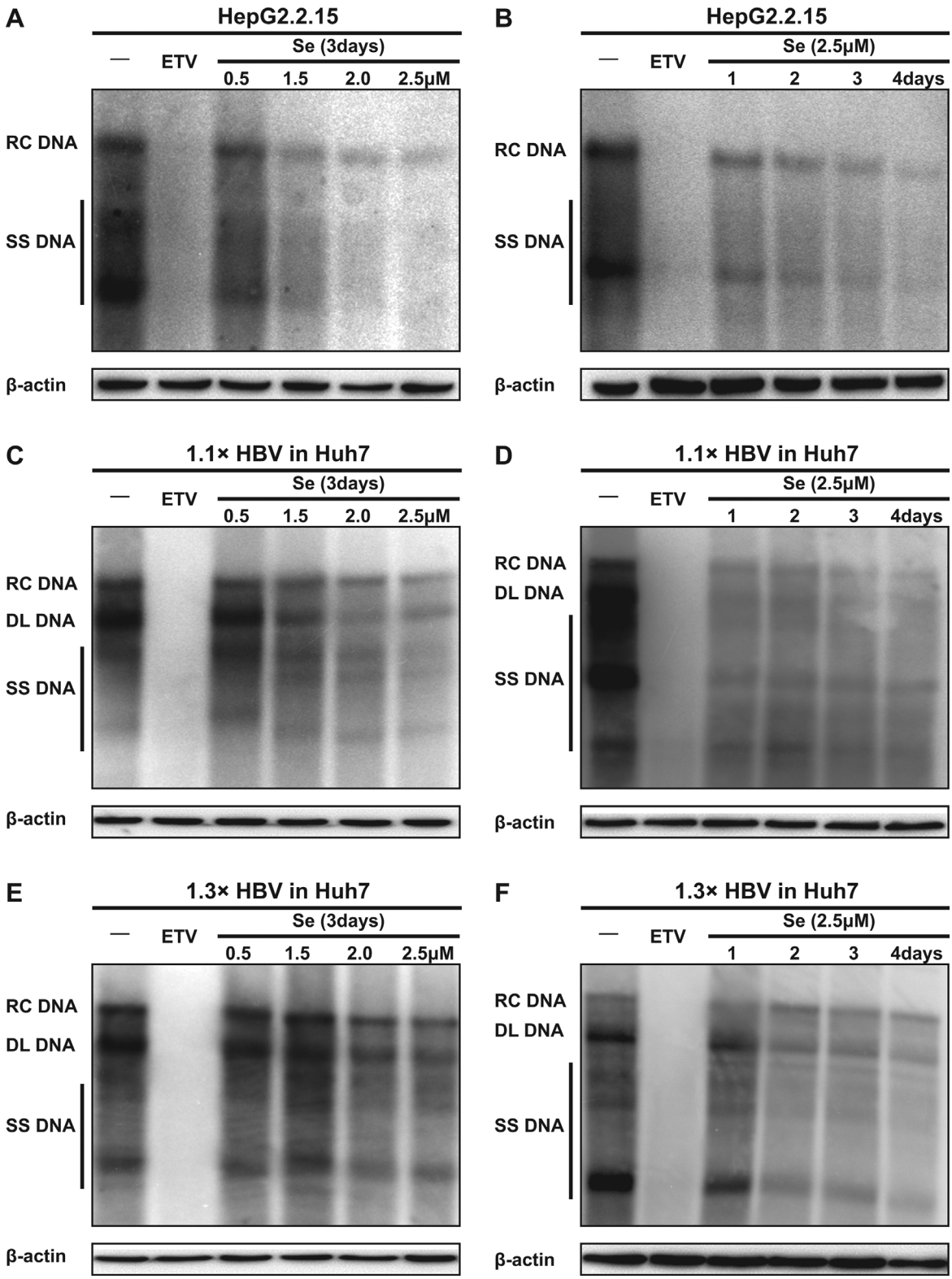

Fig. 5. Suppression of HBV genome replication by Na_2SeO_3. HepG2.2.15 and 1.1 or 1.3×HBV-transfected HuH-7 were treated with Na_2SeO_3. Partial samples were treated with various concentrations of Na_2SeO_3 and harvested on Day 3, and some samples were treated with a fixed concentration of 2.5 μM Na_2SeO_3 but harvested at different time points. Mock-treated control and 30 nM ETV-treated cells were collected on Day 4. HBV DNA levels were determined by Southern blot assay.

treatment on Day 4, as well as with 2.5 μM Na_2SeO_3 on Day 1. The HBV DNA levels then reduced progressively in line with increasing concentration and exposure time (Fig. 5A and B). In HuH-7 transfected with 1.1×HBV plasmid, Na_2SeO_3 treatment resulted in a dose-dependent inhibition of HBV DNAs, starting at 1.5 μM. Time-dependent reduction of HBV DNAs was also observed, but this was less obvious than that in HepG2.2.15 or 1.3×HBV plasmid-transfected HuH-7. This was possibly due to the profound decrease in viral DNA level observed as early as Day 1 (Fig. 5D). In 1.3×HBV plasmid-transfected HuH-7 cells, the decrease induced by Na_2SeO_3 was weaker than in the other two tested models. The reduction in HBV DNA was visible with 2.0 μM Na_2SeO_3 treatment on Day 3 (Fig. 5E) or with 2.5 μM on Day 2 (Fig. 5F).

To further investigate its anti-HBV activity, Na_2SeO_3 was compared with clinically applied nucleos(t)ide analogs, including lamivudine (3TC), entecavir (ETV), and adefovir (Ade). After three or six days' treatment with single or combine drugs, encapsidated HBV DNA levels were quantified using qPCR in HepG2.2.15. The result showed three days treatment with 2.5 μM Na_2SeO_3, 70 nM lamivudine, 9 nM entecavir, and 1.2 μM adefovir reduced viral DNA levels to 48.97 ± 2.30%, 42.16 ± 2.70%, 17.62 ± 1.22%, and 13.15 ± 0.95%, respectively (Fig. 6). On Day 6, the inhibitory effect on DNA levels were further enhanced in Na_2SeO_3 and lamivudine treated cells, with HBV DNA levels decreasing to 24.53 ± 11.09% and 24.56 ± 0.07%, respectively (Fig. 6). Moreover, combined drug treatment dramatically enhanced the inhibitory effect on HBV DNA replication. On Day 6, Na_2SeO_3 plus lamivudine, entecavir, or adefovir reduced the encapsidated HBV DNA levels to 5.49 ± 0.11%, 0.44 ± 0.04%, and 1.92 ± 0.07%, respectively (Fig. 6).

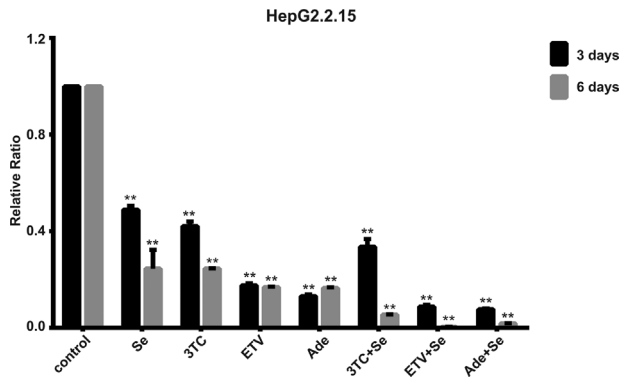

Fig. 6. Combined treatment of Na_2SeO_3 with clinically applied nucleos(*t*)ide drugs. HepG2.2.15 were treated with 2.5 μM Na_2SeO_3 (Se) alone or in combination with lamivudine (3TC, 70 nM), entecavir (ETV, 9 nM) or adefovir (Ade, 1.2 μM) for three days and six days. Intracellular DNA of samples were extracted and viral DNA was determined using qPCR. All data were normalized to the mock-treated control. $^{**}P < 0.01$.

DISCUSSION

Hepatitis B virus, a prototype member of *Hepadnaviridae* family, replicates by a unique protein-primed reverse transcription mechanism [Nassal, 2008]. Infectious virions contain the genome as a 3.2 kb relaxed circular DNA (RC DNA), which is transformed into a plasmid-like cccDNA in the host cell nucleus [Beck and Nassal, 2007]. The two subsets of viral RNAs, namely, the subgenomic RNAs (preS/S mRNAs and HBx mRNA), and the greater-than-genome-length pregenomic RNA (pgRNA) and precore mRNA, are transcribed by cellular RNA polymerase II using cccDNA as the template [Beck and Nassal, 2007]. Although Se has been reported to influence multiple virus infections [Rayman, 2000; Beck et al., 2003; Beck, 2007; Harthill, 2011], only limited information is available about whether it has any effect on HBV infection. One study has shown significantly lower concentrations of Se in serum from patients with HBV infection compared with healthy individuals [Khan et al., 2012]. Moreover, according to an eight-year follow-up investigation of 130,471 individuals in Qidong, China, Se supplementation via table salt significantly reduced the incidence of primary liver cancer [Yu et al., 1997]. However, none of the available studies showed solid evidence of the relationship between Se and HBV replication. The present study is the first to demonstrate direct inhibitory effects of Na_2SeO_3 on HBV at the cellular level, using different HBV cell models.

Both HBeAg and HBsAg are secreted to serum from HBV-infected hepatocytes, but appear at different phases of infection, and have different clinical implications [Papatheodoridis et al., 2008; Trepo et al., 2014]. HBsAg is a hallmark of infection, although HBeAg is usually associated with high levels of viral replication. Although nucleoside/nucleotide analogues can inhibit HBV replication and result in obvious reductions in HBV DNA and HBeAg levels in serum, they have little impact on HBsAg secretion [Scaglione and Lok, 2012]. The present study revealed evident declines in HBsAg/HBeAg levels with Na_2SeO_3 treatment in all three tested models (Figs. 2 and 3). However, it must be noted that the HCMV-promoter driven 1.1×HBV construct should not allow for the synthesis of HBeAg because of the lack of intact precore region at the 5' end [Nassal, 1992]. And HBeAg detected in HuH-7 transfected with 1.1×HBV plasmid might be core proteins due to antibody cross reactivity. So, intracellular core protein level was also determined in these models, and the result showed Na_2SeO_3 treatment obviously reduced core expression in host cell, but not the internal reference protein (Fig. S2). These results prompted us to further investigate the reason of the viral antigen decreases at the HBV transcription level.

Many published research articles have revealed that Se regulates various physiological activities of cells, such as gene transcription, signaling transduction, and cell apoptosis [McKenzie et al., 2002;

Zeng, 2009; Sunde and Raines, 2011]. Na_2SeO_3 was shown by northern blot assay and qPCR to inhibit HBV transcription in a dose-dependent and time-dependent manner (Figs. 4 and S1). As HBV mRNAs synthesis is primarily dependent on RNA polymerase II and the transcriptional regulation system of the host cells [Levrero et al., 2009; Quasdorff and Protzer, 2010], it is likely that the following cellular factors were possibly involved in the observed suppression.

The first potential candidate is p53. Na_2SeO_3 treatment may activate p53 by promoting its expression and phosphorylating multiple sites [Smith et al., 2004]. Both p53 and its homologue p73 have been reported to repress the activities of HBV promoters and enhancers [Lee et al., 1998; Buhlmann et al., 2008]. Hence, p53 is a possible intermediary between Na_2SeO_3 and HBV transcription.

The second possible factor is specificity protein 1 (Sp1), which binds to guanine-cytosine-rich DNA elements and regulates a wide variety of genes in mammalian cells [Chu, 2012]. At least three binding sites have been identified in the HBV genome, Sp1 activates the transcription of HBV genes by binding to these sites [Quasdorff and Protzer, 2010]. Se has been demonstrated to decrease Sp1 expression and activity [Husbeck et al., 2006], and prevent activation of Sp1-regulated HBV genes. Therefore, Se is likely to affect HBV transcription through a complicated regulatory network.

Finally, a *trans*-acting factor, HBx, encoded by the HBV genome, is a multifunctional viral protein and is broadly involved in transcription, signal transduction, cell cycle progress, apoptosis, genetic stability, and oncogenesis through protein–protein interaction [Bouchard and Schneider, 2004; Tang et al., 2006; Ng and Lee, 2011; Motavaf et al., 2013]. Tang et al. reported that both HBV transcription and replication can be affected simultaneously by HBx, but the transcriptional activation of HBx may be critical for its augmenting effect on HBV replication [Tang et al., 2006]. HBx also induces cytosolic calcium elevation and viral DNA replication, but not viral transcription, by interacting with Bcl-2 family members [Geng et al., 2012] or by activating cytosolic calcium-dependent proline-rich tyrosine kinase-2 (Pyk2) [Bouchard et al., 2001]. Although Se has not been proved to have direct effects on HBx, it has been demonstrated to affect HBx-related host factors, including p53 and nuclear factor-κB, and to result in a significant decrease in cellular calcium release [Uguz and Naziroglu, 2012]. Thus, HBx is another suspected viral factor that possibly mediates selenite-induced inhibition of HBV transcription and replication.

The Southern blot assay shown in Figure 5 showed an evident decline in HBV DNA level with Na_2SeO_3 treatment in all three tested models. However, there were dissimilarities in selenite-induced suppression in the three models, possibly due to the differences in their cell lines and HBV-expressing systems. The progressive reduction in HBV DNA displayed a similar trend to the decrease in HBV mRNAs, which indicated that one potential mechanism of Na_2SeO_3 in suppressing HBV replication is by inhibition of viral transcription. In addition to suppressing viral transcription, Se may also inhibit HBV replication by cytosolic calcium release, which is related to HBx in host cells [Bouchard et al., 2001; Geng et al., 2012]. The hypothesis that selenite inhibits HBV transcription and replication via host and viral factors still needs to be verified, and the potential involved factor (s) need to be identified by further experiments.

In addition, the anti-HBV efficacy of Na_2SeO_3 was quantitatively compared with nucleos(*t*) ide analogues using qPCR, alone or in combination (Fig. 6). On Day 6, Na_2SeO_3, in spite of a higher treating concertation, exhibited similar efficacy to the other drugs. The less potent of Na_2SeO_3 on inhibiting HBV replication compared to the other tested drugs is probably due to its indirect effect on viral polymerase. However, as Na_2SeO_3 and the nucleos(*t*) ide drugs affected different factors in the replication cycle of HBV, their combination showed more efficacy than either treatment alone did (Fig. 6).

In conclusion, these data to date, for the first time, directly prove that Na_2SeO_3 inhibits HBsAg/HBeAg expression, HBV transcription, as well as viral genome replication. This HBV suppressing effect has made Se a potential therapeutic medication to control HBV infection.

AUTHOR CONTRIBUTIONS

ZC and XZ performed the experiments. ZC, FZ, and KH designed the research. GS, GW, WS, YH, and XT provided experimental support. ZC and FZ drafted the manuscript. All authors read and approved the final manuscript for submission.

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