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

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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 immunomodulator, and controls HBV infection by stimulating the cellular antiviral cytokine expression to inhibit viral replication and by enhancing the host...
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$_2$SeO$_3$) and sodium selenate, or its organic forms, such as the amino acids selenocysteine (Sec) and selenomethionine (SeMet), and multiple selenomolecules 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, 2003] (kind gift from Dr. Yosef Shaul).

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$_2$SeO$_3$ on HBV. The data clearly clarify that application of Na$_2$SeO$_3$ 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 [Doitsch 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$^5$ 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 Na₂SeO₃ or 30 mM 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 (1×10⁴ cells/well) were cultured in 96-well plates, and Na₂SeO₃ at the indicated concentrations was added to the culture 24 hr 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 ³²P-labeled random-primed probe specific for the HBV genome. The visualized image was obtained using Cyclone Plus Storage Phosphor System (PerkinElmer, Waltham, MA), which had been dissolved in 1× sterile 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 (PerkinElmer). The cell level in the Southern blot assay was normalized to the β-actin protein level.

**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, 10⁷ cells per 10 cm plate,) or 0.6 ml (HuH-7, 3×10⁶ 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 MgCl₂ 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)**

To eliminate the possibility that any observed influences of Na₂SeO₃ on HBV replication were caused by cytotoxicity, the influence of Na₂SeO₃ on HuH-7 and HepG2.2.15 proliferation was examined using CCK-8 assay. No significant toxicity of Na₂SeO₃ for five days did not change the number of HepG2.2.15 decreased gradually, indicating cytotoxicity of Na₂SeO₃ (Fig. 1B). For both cell types, no significant differences were observed during

**RESULTS**

To eliminate the possibility that any observed influences of Na₂SeO₃ on HBV replication were caused by cytotoxicity, the influence of Na₂SeO₃ on HuH-7 and HepG2.2.15 proliferation was examined using CCK-8 assay. No significant toxicity of Na₂SeO₃ on HuH-7 cells was observed at concentrations 2.5 μM, whereas Na₂SeO₃ 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 Na₂SeO₃ for three days and 5 μM Na₂SeO₃ 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 Na₂SeO₃ (Fig. 1B). For both cell types, no significant differences were observed during

**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.

**Cytotoxicity of Na₂SeO₃ to HepG2.2.15 and HuH-7 Cells**

To eliminate the possibility that any observed influences of Na₂SeO₃ on HBV replication were caused by cytotoxicity, the influence of Na₂SeO₃ on HuH-7 and HepG2.2.15 proliferation was examined using CCK-8 assay. No significant toxicity of Na₂SeO₃ on HuH-7 cells was observed at concentrations 2.5 μM, whereas Na₂SeO₃ 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 Na₂SeO₃ treatment. Treatment with 10 μM Na₂SeO₃ for three days and 5 μM Na₂SeO₃ 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 Na₂SeO₃ (Fig. 1B). For both cell types, no significant differences were observed during

the Na$_2$SeO$_3$ treatment period at or below the safe concentration of 2.5 M for HuH-7 and 5 M 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$_2$SeO$_3$ treatment for subsequent experiments.

**Inhibition of HBsAg and HBeAg Production by Na$_2$SeO$_3$**

To study the effect of Na$_2$SeO$_3$ on HBV viral protein production, HepG2.2.15 cells or HBV plasmid-transfected HuH-7 cells were treated with Na$_2$SeO$_3$. Four concentrations, 0.5, 1.5, 2.0, and 2.5 µM, of Na$_2$SeO$_3$ 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$_2$SeO$_3$ was enough to cause a significant reduction in HBsAg and HBeAg expression compared with the mock-treated control, and Na$_2$SeO$_3$ 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$_2$SeO$_3$ failed to decrease HBsAg and HBeAg levels. A dose-dependent suppression was observed, starting from 1.5 µM Na$_2$SeO$_3$ (Fig. 2C and D). Viral antigen in 1.3× HBV plasmid-transfected HuH-7 cells showed even stronger resistance to Na$_2$SeO$_3$. HBsAg and HBeAg levels started to decline with 2 µM Na$_2$SeO$_3$, and the decline was further enhanced by 2.5 µM Na$_2$SeO$_3$ treatment (Fig. 2E and F).

Next, a fixed concentration (2.5 µM) of Na$_2$SeO$_3$ was used to assess viral antigens expression over a time course. For HepG2.2.15 cells, 2.5 µM Na$_2$SeO$_3$ 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$_2$SeO$_3$ treatment had caused a reduction of 62.81 ± 2.59% and 54.69 ± 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$_2$SeO$_3$ treatment in HuH-7 cells transfected with 1.1 or 1.3× HBV plasmid produced a drop in HBsAg level to 41.16 ± 10.55% and 42.14 ± 10.15% (Fig. 3C and E), respectively, and a drop in HBeAg level to 29.73 ± 8.33% and 32.32 ± 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$_2$SeO$_3$**

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$_2$SeO$_3$. Four concentrations, 0.5, 1.5, 2.0, and 2.5 µM, of Na$_2$SeO$_3$ 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$_2$SeO$_3$ treatment, but with slight differences.

In HepG2.2.15 cells, 0.5 µM Na$_2$SeO$_3$ 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$_2$SeO$_3$ treatment (Fig. 4A). When the concentration was fixed at 2.5 µM, progressive repression by Na$_2$SeO$_3$ of HBV
Fig. 2. Dose-dependent suppression of HBsAg/HBeAg expression by Na$_2$SeO$_3$. HepG2.2.15 and 1.1× or 1.3× HBV-transfected HuH-7 cells were treated with Na$_2$SeO$_3$ 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 Na₂SeO₃. HepG2.2.15 and 1.1× or 1.3× HBV-transfected HuH-7 cells were treated with 2.5 μM Na₂SeO₃ or 30 nM ETV. Supernatants of the Na₂SeO₃-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.
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 Na₂SeO₃ treatment in dose-dependent and time-dependent manners, which was reduced to 49.28 ± 3.82% in the presence of 2.5 μM Na₂SeO₃ for four days. In HuH-7 cells transfected with 1.1×HBV plasmids, there was a dose-dependent suppression of viral mRNA by Na₂SeO₃ (Fig. 4B). In addition, perceptible repression of HBV transcription was observed from Day 2 of 2.5 μM Na₂SeO₃ treatment, which also occurred in a time-dependent manner (Fig. 4B). Na₂SeO₃-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 Na₂SeO₃, and progressed in a dose-dependent manner. Na₂SeO₃ 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 Na₂SeO₃ 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 Na₂SeO₃ influence specifically HBV transcription and expression.

**Suppression of HBV DNA Replication by Na₂SeO₃**

The fact that Na₂SeO₃ 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 Na₂SeO₃ 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, Na₂SeO₃ 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 Na₂SeO₃ resulted in a very obvious repression of HBV DNA in HepG2.2.15 cells. Clear DNA decline occurred with 0.5 μM Na₂SeO₃....
Fig. 5. Suppression of HBV genome replication by Na₂SeO₃. HepG2.2.15 and 1.1 or 1.3×HBV-transfected HuH-7 were treated with Na₂SeO₃. Partial samples were treated with various concentrations of Na₂SeO₃ and harvested on Day 3, and some samples were treated with a fixed concentration of 2.5 μM Na₂SeO₃ 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 Na2SeO3 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, Na2SeO3 treatment resulted in a dose-dependent reduction 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 Na2SeO3 was weaker than in the other two tested models. The reduction in HBV DNA was visible with 2.0 μM Na2SeO3 treatment on Day 3 (Fig. 5E) or with 2.5 μM on Day 2 (Fig. 5F).

To further investigate its anti-HBV activity, Na2SeO3 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 Na2SeO3, 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 Na2SeO3 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, Na2SeO3 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).

**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 Na2SeO3 on HBV at the cellular level, using different HBV cell models.

Both HBsAg 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 Na2SeO3 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 Na2SeO3 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_{2}SeO_{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 regulatory 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_{2}SeO_{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_{2}SeO_{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_{2}SeO_{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_{2}SeO_{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_{2}SeO_{3} was quantitatively compared with nucleoside(t)ide analogues using qPCR, alone or in combination (Fig. 6).

On Day 6, Na_{2}SeO_{3}, in spite of a higher treating concentration, exhibited similar efficacy to the other drugs. The less potent of Na_{2}SeO_{3} on inhibiting HBV replication compared to the other tested drugs is probably due to its indirect effect on viral polymerase. However, as Na_{2}SeO_{3} and the nucleoside(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_{2}SeO_{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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