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Optimization of an Efficient Cell Culture Hepatitis B Infection System for Assessment of Hepatitis B Virus Neutralizing Monoclonal Antibodies

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ABSTRACT

Background: Human polyclonal plasma-derived hepatitis B immunoglobulin (HBIG) is currently used for immunoprophylaxis of HBV infection. The development of virus-neutralizing monoclonal antibodies (MAbs) requires the use of optimized cell culture systems supporting HBV infection.

Objective: This study aims to optimize the hepatitis B virus infectivity of NTCP-reconstituted HepG2 (HepG2-NTCP) cells to establish an efficient system to evaluate the HBV-neutralizing effect of anti-HBs MAbs.

Methods: Serum-derived HBV (sHBV) and cell culture-derived HBV (ccHBV) were simultaneously used for the optimization of HBV infection in HepG2-NTCP cells by applying different modifications.

Results: Our results for the first time showed that in addition to human serum, monkey serum could significantly improve ccHBV infection, while fetal and adult bovine serum as well as duck and sheep serum did not have a promotive effect. In addition, sHBV and ccHBV infectivity are largely similar except that adding 5% of PEG, which is commonly used to improve in vitro infection of ccHBV, significantly reduced sHBV infection. We showed that a combination of spinoculation, trypsinization, and also adding human or monkey serum to HBV inoculum could significantly improve the permissivity of HepG2-NTCP cells to HBV infection compared with individual strategies. All anti-HBs MAbs were able to successfully neutralize both ccHBV and sHBV infection in our optimized in vitro system.

Conclusion: Our study suggests different strategies for improving ccHBV and sHBV infection in HepG2-NTCP cells. This cell culture-based system allows assessment of HBV neutralizing MAbs and may also prove to be valuable for the analysis of other HBV neutralizing therapeutics.

Keywords: Hepatitis B virus, HepG2-NTCP cells, HBs antigen, HBe antigen, cccDNA, rcDNA

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INTRODUCTION

Hepatitis B virus is a hepatotropic enveloped DNA virus of the Hepadnaviridae family (1). It is estimated that more than 250 million individuals are chronically infected with this virus worldwide of whom almost a third are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) (2, 3).

Since HBV's discovery in late 1960, there is no cure yet for this infection, which is partially due to the lack of reproducible and robust cell culture systems that mimic the viral life cycle (4, 5). Thus, developing an efficient and reproducible cell culture system suitable for studying mechanisms involved in HBV infection, screening novel antiviral strategies, and also assessing the neutralizing efficacy of anti-HBs antibodies is necessary.

Primary human hepatocytes (PHHs), Primary Tupaia hepatocytes (PTHs), and HepaRG cells are susceptible to HBV infection and serve as available systems for HBV infection (6). However, preparation of PHH due to its limited supply, variation in the genetic background from donor to donor, and high cost limits its application (7, 8). In this regard, HepaRG cells have advantages over PTHs and PHHs, but still suffer from limitations, such as time-consuming cell proliferation, and the need for 2 weeks differentiation step before infection (9, 10). The recent identification of bile-acid pump NTCP, as a bona fide receptor of HBV, was a major milestone in HBV research and has opened the door to establishing NTCP-overexpressing hepatoma cell lines like HepG2 and Huh7, which are susceptible to HBV infection (11, 12). Unfortunately, the average percentage of infected NTCP-reconstituted cells remains low and the infection of these cells is not efficient enough; thus, further optimization and standardization are necessary to improve the HBV infection in these cells (11, 13-16). Although recent studies have reported the development of novel HepG2-NTCP subclones or the application of strategies that support long-term viral spread, still a high viral load is needed to efficiently infect HBV infection susceptible cell lines (13, 17). The effects of some modifications to improve the HBV infection sensitivity of HepG2-NTCP cells have recently been evaluated and promising results have been reported. However, there is relatively poor knowledge of the different features of infectivity patterns of serum-derived HBV (sHBV) and cell culture-derived HBV (ccHBV) simultaneously used in vitro. Most of the previous studies trying to optimize and enhance HBV infectivity in vitro have only used one source of the virus, mostly ccHBV. Therefore, we used both sHBV and ccHBV during all modifications used for the optimization of HBV infectivity to compare the infectivity pattern of these two sources of the virus. Spinoculation, centrifugation of HepG2-NTCP cells during HBV inoculation, is one of these modifications that is shown to significantly enhance virus infectivity. The effect of this strategy has been only evaluated on ccHBV infection, but not on the sHBV viral preparation (15). The other strategy that is shown to improve HBV infectivity is adding HBV inoculum to suspended NTCP-HepG2 cells. This modification can improve the virus infectivity by disrupting the junctions between cells and exposing more NTCP to HBV particles. The efficiency of this strategy has been evaluated with both ccHBV and sHBV inoculums (18).

For many years PEG has been used to enhance HBV infection in different cell culture systems (11, 14, 17, 19, 20). Given the importance of PEG, a recent study has shown that in addition to adding PEG during HBV inoculation, maintaining PEG in a culture medium until 13 days post-infection improved the rate of infection by both ccHBV and sHBV and, most importantly, this strategy promoted virus spread. Another modification that has been reported to improve HBV infectivity is adding heparin at a physiological concentration to the virus inoculum (21).

Although these modifications have been made so far, it has been reported that still large quantities of the virus are needed to

infect NTCP-expressing hepatoma cell lines, and the average percentage of HBsAg positive cells remains low. Therefore, further optimization to improve HBV infection in NTCP-reconstituted cell lines is required.

In the present study, we improved the permissivity of HepG2-NTCP cells to two different sources of HBV by establishing simultaneous modifications, including adding a normal human serum to virus inoculum, spinoculation, and suspended cell conditions. We reasoned that there might be a soluble factor in the serum of human, as a susceptible species to the HBV infection, and rhesus monkey, as a phylogenetically close species to human but non-susceptible to the infection, that facilitate the infection. For the first time, we also evaluated the effect of different species of serum other than humans, such as monkey, duck, sheep, fetal, and adult bovine serum on improving the virus infection. This is the first comprehensive study that evaluated the effect of different modifications and different species of serum on serum-derived and also cell culture-derived HBV simultaneously to determine whether the combination of these modifications has a synergistic effect or whether these two sources of the virus show different features at the presence of different modifications. Finally, the optimized system was used to assess the neutralizing efficacy of anti-HBs murine and chimeric MAbs on ccHBV and sHBV viral particles.

MATERIALS AND METHODS

Cell culture. HepG2 cells stably transfected with human NTCP (HepG2-NTCP) that are susceptible to HBV infection at high infection rates (22) were used in this study. HepG2-NTCP cells were cultured in Dulbecco's modified Eagle medium (DMEM-low glucose, Gibco, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, USA), non-essential amino acids (Gibco, UK) and 30µg/ml Blasticidin (Gibco, USA) at 37˚C in 5% CO_2 humidified incubator. HepAD38 cells (Established by Professor Christoph Seeger from the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia) were cultured in DMEM/F12 (Gibco, UK) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and non-essential amino acids.

Preparation of HBV inoculum. HepAD38 cells, which are capable of stably producing high titers of *ayw* subtype HBV (genotype D) (23), were cultured on collagen-coated flasks in DMEM-F12 media in the absence of tetracycline, and the medium was changed every 3 days until the cells got 90% confluency. Then, the supernatant was removed and fresh media was added to the cells. After 3 days, the supernatant was collected for virus concentration. After collection, the medium was centrifuged at 2500g at 4°C and passed through a 0.22 µm filter (Millipore, USA) to remove any cell debris. In the end, virus particles were precipitated and concentrated almost 120-fold in the presence of PEG 8000 (Sigma,) (final concentration 8%). Briefly, after adding PEG to the culture media containing the virus, it was completely mixed at low rotation speed for 1 hour, and then the mixture was incubated at 4 degrees without stirring, followed by centrifugation at 4500g for 30 min, and then the pellet was resuspended in PBS. The concentrated virus stock was aliquoted and stored at −80°C, and the HBV genomes/ml was determined with a TaqMan-based qPCR method (Gene proof, Czech Republic).

Pooled sera from unidentified HBV-infected individuals containing high HBV viral load (4. 7×10^9 copy/ml) were also used to infect cells similar as the HepAD38-derived HBV stock. The quantity of HBV DNA in pooled sera was also determined by the HBV qPCR TaqMan kit (Gene proof). Both ccHBV and sHBV isolates we used in our study belonged to genotype D.

HBV infection. HepG2-NTCP cells were cultured in 24-well collagen-coated plate with a density of 5×10^4 cells/well for 3 days. To

differentiate HepG2-NTCP cells, which is necessary for successful HBV infection, the cells were cultured in a DMEM differentiation medium containing 1.74% DMSO and 1% FBS. The cells were incubated in this differentiation medium during the virus inoculation and afterward. After 2 days of differentiation, the cells were infected with the concentrated HBV at indicated MOI: 100, 200, 400, or 800 genome equivalents per cell (GEq/cell). HBV infection of differentiated HepG2-NTCP cells was performed in a DMEM differentiation medium supplemented with the indicated concentration of PEG 8000 (1.25, 2.5, 5, and 10%) for 16 hours. Following removing the HBV inoculum, cells were washed 3 times with PBS, and fresh media was added to each well. Cells were incubated for 12 more days and media was exchanged every 4 days. On day 13, after collecting the supernatant of cells, their DNA was also extracted.

To perform spinoculation, immediately after sHBV or ccHBV inoculum was added to differentiated HepG2-NTCP cells, the plate was centrifuged at room temperature at 1000g for 60 min and then transferred to a 37°C CO2 incubator. After 16 hours of incubation, the inoculum was removed and cells were washed 3 times and maintained in a differentiation medium like other cells.

In the case of infection on suspended cells, differentiated HepG2-NTCP cells were harvested using 0.25% trypsin/ethylene diamine tetra acetic acid (EDTA) solution (Sigma, USA), and then washed once with a DMEM medium containing 10% FBS. The cells were then suspended in the differentiation medium (1.74% DMSO and 1% FBS) that contained HBV inoculum and indicated concentration of PEG 8000, and were seeded into 24-well collagen-coated plate at a density of 12×10^5. After 16 hours of incubation, the inoculum was removed and the cells were washed 3 times and maintained in a differentiation medium like the other cells.

To evaluate the effect of human or other species serum on the HBV infection, the final concentration of all the species' serum was 5% in differentiation medium which contained 1.74% DMSO and 1% FBS).

In all the experiments, Myrcludex-B (Myr-B), a synthetic lipopeptide derived from the preS1 domain of the HBV envelope protein (24), was used to inhibit HBV infection. Myr-B was incubated at the final concentration of 700 nM with cells for 30 minutes before infection and then the medium was replaced with fresh medium containing HBV inoculum with the same concentration of Myr-B. After 16 hours of incubation and washing the inoculum, the cells were maintained in the differentiation medium, without Myr-B, like the other cells.

Assays for evaluating permissivity of HepG2-NTCP cells to HBV. The establishment of HBV infection in HepG2-NTCP cells was determined by measuring the secretion of HBsAg and HBeAg in supernatant of infected cells on 5, 9 and 13 day post infection (dpi), and also cccDNA and HBV rcDNA levels in the lysate of infected cells at 13dpi. The measurement of HBeAg was carried out by commercial ELISA kit according to the manufacture's protocol (Dia. Pro kit, HBE.CE, Italy). For HBsAg detection, 5ug/ml of two affinity purified anti-HBs monoclonal antibodies (mAbs) designated 4G4 and 6E3, previously generated and characterized in our lab (25-27), were coated on ELISA plate, and incubated overnight. After blocking with 1% bovine serum albumin (BSA), supernatant of infected cells were added and incubated at 37 °C for 1h. After washing, biotin-conjugated anti-HBs mAb (Enzygnost HBsAg 6.0, SIEMENS, Germany) was added and incubated at 37°C for 1 h. Following the washing, streptavidin-HRP-conjugate (Enzygnost HBsAg 6.0) was added and incubated at 37°C for 1h. After washing and adding 3, 3', 5, 5'- Tetramethylbenzidine (TMB) substrate (Pishtaz Teb, Karaj, Iran), optical density (OD) was measured at 450 nm. For each experiment, the OD values were measured using 50 ul of culture supernatant and the OD of the sample/cutoff (S/CO) was

calculated by comparing with the relevant negative control.

To evaluate cccDNA and rcDNA, the total DNA was extracted from infected HepG2-NTPC cells by either High Pure PCR Template Preparation Kit (Roche, Germany) or Microspin columns (Macherey-Nagel, Dueren, Germany) according to the manufacture's protocol. Quantitative Real-time PCR (qPCR) was performed using SYBER Green Master Mix (Qiagen, Germany) and StepOne Plus Real-Time PCR System (Applied BioSystems, USA). The genes were normalized to Prnp gene (Prion Protein, as internal control). To avoid false-positive detection of cccDNA due to the presence of HBV DNA molecules, extracted DNA was treated with T5 exonuclease (NEB, Germany) before further analysis under the following conditions: 8.5ul of extracted DNA was incubated with 5 unit of T5 exonuclease for 2 h at 37°C and was subsequently inactivated by incubation at 95°C for 2 minutes. After T5 exonuclease treatment, 30ul of distilled water was added to each sample to reach 4-fold dilution and the samples were quantified by qPCR. HBV DNA and also housekeeping gene was measured in untreated samples simultaneously.

The primers sequences used for amplification of Prnp, rcDNA and cccDNA were as follows: Prnp-forward: 5′- TGCTGGGAAGTGCCATGAG-3′ and Prnp-reverse: 5′-CGGTGCATGTTTTCACGATAGTA- 3′, rcDNA-forward: 5′ -GGAGGGATACATAGAGGTTCCTTGA-3′ and rcDNA-reverse 5′-GTTGCCCGTTTGTCCTCTAATTC- 3′, and HBV cccDNA-forward: 5′- CCGTGTGCACTTCGCTTCA- 3′ and HBV cccDNA reverse: 5′-GCACAGCTTGGAGGCTTGA- 3′. All experiments were performed with one stock of ccHBV or sHBV inoculum.

Assessment of HBV Neutralization by anti-HBs MAbs

Previously generated and characterized murine, 4G4, 6C1, 5F9 and chimeric human-murine, c-4G4, anti-HBs MAbs (27) were used in this study. 20 µg/ml of the MAbs was pre-incubated for 3h at 37°C with sHBV and ccHBV inoculums (multiplicity of infection (MOI)= 400) and subsequently added to the differentiated HepG2-NTCP. After overnight incubation, the cells were washed three times with PBS and maintained in differentiation medium until 13 days after the infection. Quantification of HBeAg was performed by ELISA at 13dpi in the supernatant of infected cells as described above. Quantification of cccDNA in cell lysates of the infected cells at 13 dpi was performed by q-PCR as described above. 0.1 IU/ml of commercial polyclonal anti-HBs antibody (HBIG, KEDRION, Italy) was used as a control.

Immunofluorescence Assay for HBsAg Detection

HepG2-NTCP cells were cultured in a 24-well collagen-coated plate, and after differentiation, the cells were infected with the ccHBV and sHBV at MOI 400. At 13dpi, cells were washed with PBS for 2 times, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (RT) and permeabilized with 0.5% saponin in PBS for 10 min at RT. After washing once with PBS, the cells were blocked with 5% sheep serum in 0.1% saponin for 2h at RT, and then incubated with 5ug/ml anti-HBs mAb 5F9, previously generated and characterized in our lab)diluted in blocker) (25-27). After overnight incubation at 4˚C, the cells were washed three times with 0.05% PBS-Tween 20 (PBS-T), 5 min each wash. FITC-labeled sheep anti-mouse Ig secondary antibody (Sina Biotech, Tehran, Iran) diluted in 2% sheep serum in 0.1% saponin was added to the cells, and then the cells were incubated at RT for 2 h. After washing three times with PBS-T, the stained cells were observed by invert fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using

GraphPad Prism 5 and One-way ANOVA and post-hoc multiple comparisons Scheffe test and independent Student's t-test. For all tests, P values less than 0.05 were considered as statistically significant. (P<0.05 (*), P<0.01 (**) and P<0.001 (***)).

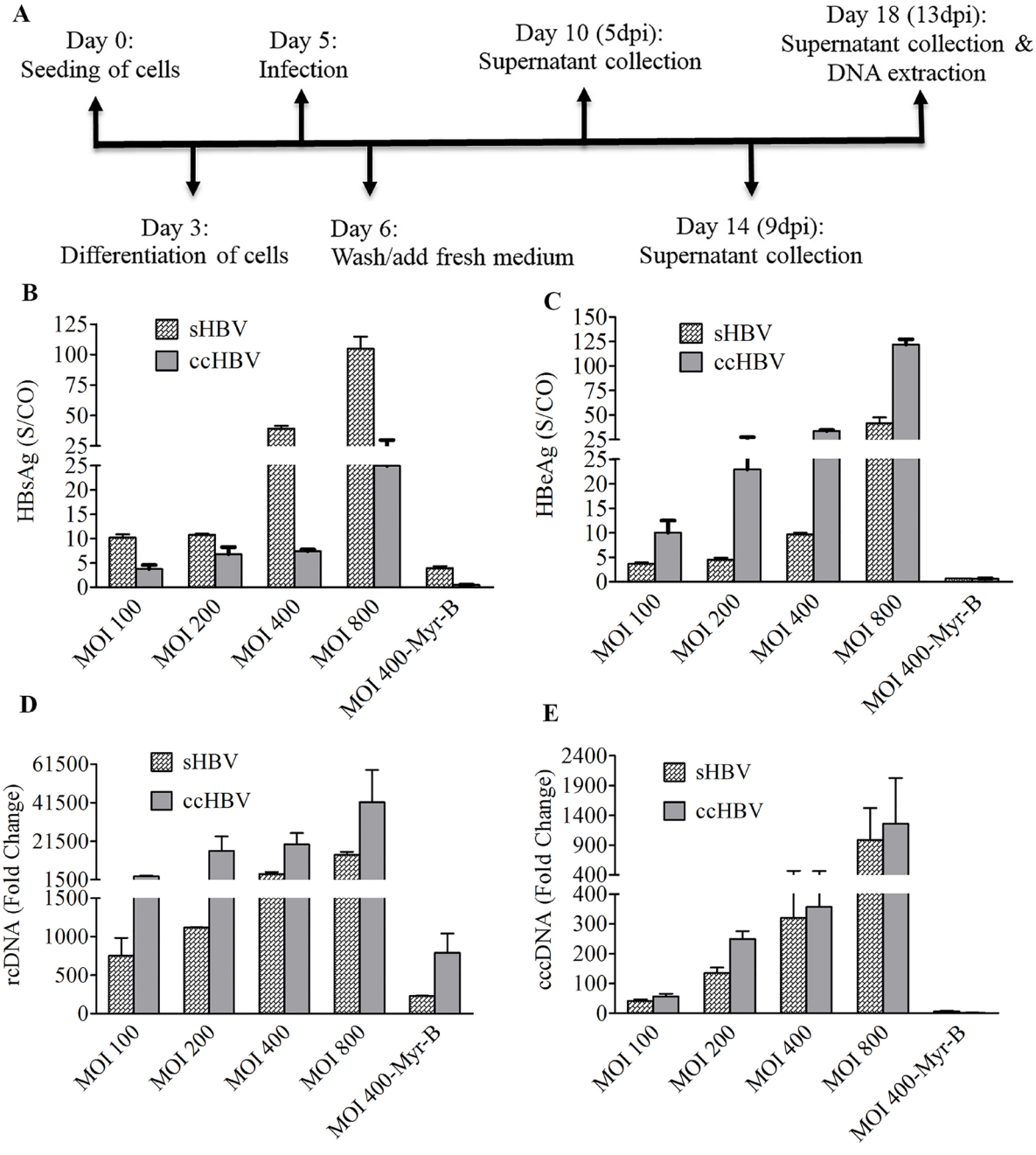

Figure 1. Establishing HBV infection in HepG2-NTCP cells with sHBV and ccHBV. Differentiated HepG2-NTCP cells were infected with different MOIs of sHBV and ccHBV inoculums in the presence of 2.5% PEG. After 16h, the virus inoculum was removed and the cells were washed 3 times with PBS and a fresh differentiated medium containing 1% FBS and 1.74% DMSO was added. The cell supernatant was exchanged every 4 days until 13 dpi. Infection in the presence of 700 nm Myr-B was performed as a control. (A) Schematic illustration of experimental design. (B, C) HBsAg and HBeAg were detected by ELISA in the supernatant of infected cells at 13dpi, and (D, E) HBV rcDNA and cccDNA were evaluated by q-PCR in the lysate of infected cells at 13dpi. HBV rcDNA and cccDNA were normalized to the Prnp gene and were plotted as a fold change relative to the control group that had not been infected with HBV. Results represent the mean (± standard deviation, SD) data from duplicate independent experiments. The threshold value for HBsAg and HBeAg positivity was defined as an optical density (OD) of the sample/cutoff (S/CO) ratio of >1. Myr-B: Myrcludex-B, S/CO value: sample/cutoff.

RESULTS

HepG2-NTCP cells are infectable by both sHBV and ccHBV. Since it has been previously reported that NTCP is inefficient to mediate infection by sHBV (28), we first addressed this issue in HepG2-NTCP cells. The level of infectivity of different MOIs of two sources of HBV inoculums, including ccHBV and sHBV, was compared in the differentiated HepG2-NTCP cells. Pooled serum of chronic HBV patients with 4.7×10^9 virus copy/ml and ccHBV with 7.3×10^9 copy/ml were used as viral source to infect HepG2-NTCP cells. After 2 days of culturing HepG2-NTCP cells with differentiation medium, they were infected with MOI 100, 200, 400, and 800 of sHBV and ccHBV. Due to the importance of PEG in efficient in vitro infection of HBV, 2.5% PEG in the virus inoculum was included. The procedure of infection design is shown in Figure 1A. Our results showed that inoculating viremic serum samples directly to HepG2-NTCP cells was able to successfully establish HBV infection even at MOI100 (Figures 1B, C, D, E). HBsAg expression on ccHBV and sHBV infected HepG2-NTCP cells at MOI400 at the end of experiment (13 dpi) was also evaluated by Immunofluorescence assay (IF) using anti-HBs monoclonal antibody (Figure 2). A diffuse intracellular and membrane labeling was observed in both ccHBV and sHBV infected HepG2-NTCP. The percentage of infected cells after infection with sHBV was slightly higher than that with ccHBV.

However, the results of HBeAg and HBV DNA showed that ccHBV could establish higher levels of infection in HepG2-NTCP cells compared with sHBV at the relevant MOIs (Figures 1C, D). However, cccDNA level did not show a consistent result, and each MOI in two different inoculums showed different patterns (Figure 1E). According to HBeAg, ccHBV was much more infectious that sHBV (at least 3 times) in all MOIs; however, the results of cccDNA and rcDNA did not show such a high difference among the infectivity level of the two different sources of the virus.

Since there is a possibility that HBsAg or viruses remaining from the inoculum contribute to the HBsAg signal, we included a control that contained Myr-B. 700 nM of Myr-B during the inoculation step successfully prevented both ccHBV and sHBV infection in all experiments (Figures 1B, C, D, E). In addition, the concentration of HBsAg in both inoculums was determined by ELISA and the results showed that sHBV and ccHBV inoculums contained 2.9 and 0.03 mg/ml HBsAg, respectively. Therefore, DNA/HBsAg in sHBV and ccHBV was 1.6×10^9 and 2.4×10^{11}, respectively.

The effect of PEG on ccHBV infectivity was more significant than sHBV infectivity. Conditions for optimal infection with the two different sources of HBV at MOI 400

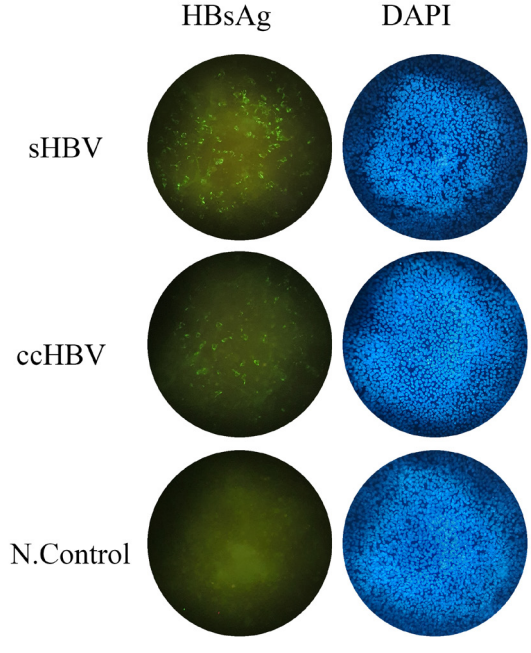

Figure 2. Immunofluorescence staining of HBsAg in cells infected with sHBV and ccHBV. No significant differences in cellular expression of HBsAg between sHBV and ccHBV-infected HepG2-NTCP cells were observed. HepG2-NTCP cells cultured in 24-well collagen-coated plates were infected with MOI 400 of ccHBV and sHBV. At 13dpi, immunofluorescence staining was performed with anti-HBs mAb (5F9). Neg. Control: uninfected HepG2-NTCP cells stained with 5F9 mAb.

were evaluated by assessing the effect of different concentrations of PEG8000 during infection. MOI 400 of both sHBV and ccHBV in the absence or presence of 1.25, 2.5, 5, 10% PEG were used to infect HepG2-NTCP cells. After an overnight incubation, the cells were washed 3 times with PBS to remove viruses and PEG, and maintained in fresh differentiation medium lacking PEG for the rest of the experiments. Our results showed that PEG improved the infection efficiency of ccHBV in a dose-dependent manner, and adding 5% PEG to ccHBV inoculum resulted in the highest level of infection, while increasing the concentration of PEG to 10% had an inhibitory effect on ccHBV infectivity (Figures 3A, B, C, D, E, F, G, H). Although the infectivity of sHBV seemed to be PEG independent, 2.5% PEG could improve the level of cccDNA during sHBV infection, while it did not affect the other infection markers after sHBV infection (Figure 3G). On the other hand, adding 5 to 10% of PEG in sHBV inoculum had a reverse effect and inhibited sHBV infectivity in HpeG2-NTCP cells. Nevertheless, adding 10% PEG caused a significant increase in HBsAg (Figure 3A) compared with 5% PEG or no PEG conditions, while based on HbeAg (Figure 3C), 10% of PEG leads to a significant reduction in sHBV infection. Therefore, we assumed that the high level of HbsAg in the presence of 10%PEG after sHBV infection was not the result of infection and was likely a false positive result.

Kinetics of HBsAg and HBeAg secretion after ccHBV and sHBV infection. Both HBsAg and HBeAg are routinely used as sensitive infection seromarkers of HBV infection. We investigated the pattern of their secretion during 26 days of infection with ccHBV and sHBV in HepG2-NTCP cells. Our results showed that sHBV and, to a lesser extent, ccHBV inoculums contained large amount of HBsAg (Figures 4A, B). Therefore, the total amount of HBsAg at 5 dpi in the supernatant of HepG2-NTCP infected with both sHBV and ccHBV was not the result of established infection, since the negative control that contained Myr-B still showed high level of HBsAg at this time point. The same pattern was observed at 9 dpi with sHBV, and to a lesser extent for ccHBV. Therefore, HBsAg is not a reliable marker at the early stage of sHBV and ccHBV infection. Additionally, at 13 dpi, almost all of the HBsAg in the supernatant of HepG2-NTCP cells infected with ccHBV was the result of established infection, since the negative control that contained Myr-B showed a negligible level of HBsAg (S/CO: 1.1) at this point. In contrast, sHBV inoculum contained high level of HBsAg from the subviral particles which were detected even 13 dpi in the supernatant of the infected cells. Therefore, high level of HBsAg in the supernatant of the cells infected with sHBV inoculum even after 13 dpi was not entirely the result of the established HBV infection, since the negative control that contained Myr-B showed a noticeable level of HBsAg (S/CO: 7.5) at 13 dpi. While HBsAg could be secreted from infected cells or be associated to subviral particles, our results showed that the secretion of HBeAg was the results of established infection with both ccHBV and sHBV at 5, 9, and 13 dpi (Figures 4C, D). HBsAg/HBeAg ratio following ccHBV infection was excessively lower than after sHBV infection at all the tested points (Figure 4E). As Figure 4A shows, even at 26 days after sHBV infection, HBsAg (S/CO) of negative control that contained Myr-B was 2.9, while this value at the same point after ccHBV infection was 0.75.

Enhancement of HBV infectivity by different modifications. Some studies have shown that trypsinization of HepG2-NTCP cells, spinoculation, and adding healthy human plasma improves HBV infection (21, 29, 30). Therefore, we studied the effect of trypsinization, spinoculation, and also adding healthy human serum (instead of plasma) on HBV infection individually and in combination. The control group for all the modifications was adherent monolayer differentiated HepG2-NTCP cells

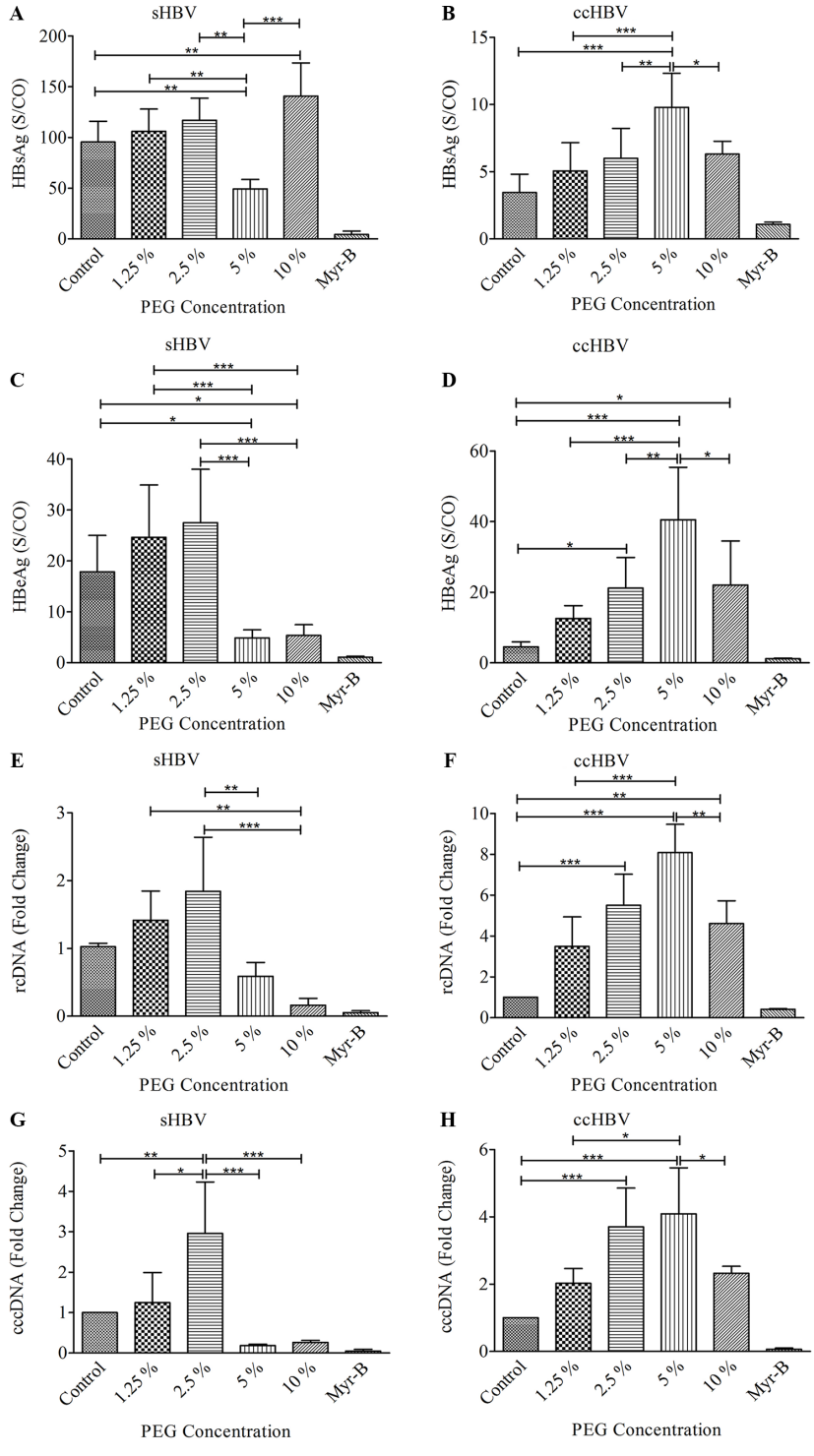

Figure 3. The effect of PEG on ccHBV infectivity was more significant than on sHBV infectivity. HepG2-NTCP cells were infected with MOI 400 of sHBV and ccHBV inoculum in the absence or presence of 1.25, 2.5, 5, and 10% PEG 8000. After overnight incubation, cells were washed 3 times with PBS to remove viruses and PEG and maintained in fresh differentiation media that did not contain PEG for the rest of the experiments. (A, B) Levels of HBsAg and (C, D) HBeAg were detected by ELISA in the supernatant of cells at 13 dpi. (E, F) rcDNA and (G, H) cccDNA expression levels were detected by q-PCR in the lysate of infected cells at 13dpi. rcDNA and cccDNA were normalized to the Prnp gene and were plotted as a fold change relative to the control group. Control: HBV infection in the absence of PEG. Data are shown as mean ± SD of at least three independent experiments. *P<0.5, **P<0.01, ***P<0.001, for statistical analysis. The threshold value for HBsAg and HBeAg positivity was defined as an OD of the sample/cutoff (S/CO) ratio of >1.

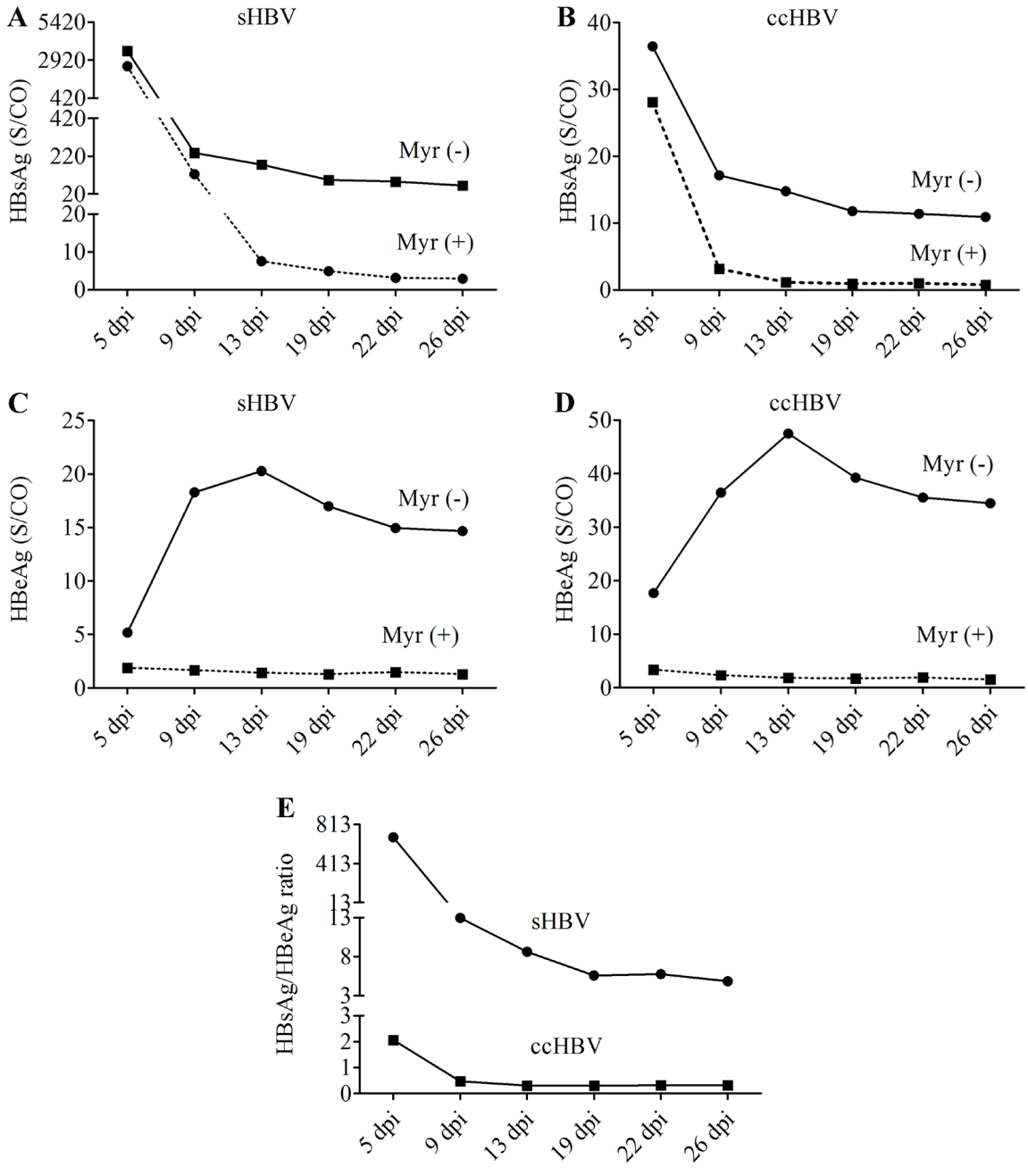

Figure 4. Kinetic of HBsAg and HBeAg secretion after ccHBV and sHBV infection in HepG2-NTCP cells. HepG2-NTCP cells were infected with MOI 400 of sHBV and ccHBV in the presence of 2.5% PEG and the presence or absence of 700nM Myr-B. The supernatant of cells was harvested on different days post-infection and HBsAg (A, B) and HBeAg (C, D) were measured by ELISA. (E) HBsAg/HBeAg ratio in the supernatant of infected cells was calculated at different time points after infection.

that have been infected with MOI400 in the presence of 2.5 and 5% PEG 8000 for sHBV and ccHBV infection, respectively.

Cell trypsinization and suspension improve virus infectivity. To study the effect of suspended cell condition on HBV infection in HepG2-NTCP cells, the differentiated HepG2-NTCP cells were suspended by trypsin-EDTA and then the sHBV and ccHBV inoculums at MOI400 were added to the suspended cells pellet in the presence of 2.5 and 5% PEG 8000 for sHBV and ccHBV, respectively, and the results were compared with the control one. It is noteworthy that the cells were detached after trypsin-EDTA treatment by gentle pipetting with no scraping.

Our results showed that trypsinization of HepG2-NTCP cells and preparing cell suspension before HBV inoculation could significantly increase the infection mediated by both sHBV and ccHBV inoculums at a similar order (Figures 5A-H). To assure that the effect of suspended cell condition on the infectivity of sHBV and ccHBV is NTCP-dependent and not through an alternative way to enter the cells, an extra control group of suspended cells that contained Myr-B (tryp-Myr-B) was included. Since Myr-B-treated suspended cell control (Tryp-Myr-B (showed no significant increase in any infection markers we tested, we assumed that this increase in HBV infectivity following trypsin-EDTA treatment is NTCP-dependent and is through the authentic HBV entry pathway. Our results showed that suspended condition significantly increased HBsAg (Figures 5A, B) and HBeAg (Figures 5C, D), indicating successful mRNA transcription from the established cccDNA during the infection. Similar to the enhanced HBsAg and HBeAg expression, HBV rcDNA (Figures 5E, F) and also cccDNA (Figures 5G, H) levels increased under optimized conditions, suggesting successful establishment of both sHBV and ccHBV infections after the suspended condition.

Spinoculation showed a weak effect on both sHBV and ccHBV infectivity. To evaluate the effect of spinoculation on infection, differentiated HepG2-NTCP cells were centrifuged after adding HBV inoculums at MOI 400 in the presence of 2.5% and 5% PEG 8000 for sHBV and ccHBV infection, respectively, and the results were compared with the control one. Based on our result, the effect of spinoculation on both ccHBV and sHBV infectivity was weak. In particular, spinoculation could not increase the level of secreted HBsAg (Figure 5B) and HBeAg (Figure 5D) after the infection with ccHBV in HepG2-NTCP cells; however, there was a significant elevation in HBV rcDNA (Figure 5F) but not cccDNA (Figure 5H) after the infection with ccHBV. In addition, the effect of spinoculation on the infectivity of sHBV was more significant than that of ccHBV and it lead to a significant increase of HBV rcDNA and cccDNA. (Figures 5E, G).

Healthy Human and Monkey Serum Contain Components that Enhance HBV Infectivity

Since it has been previously shown that heparin at physiologic concentration in healthy human plasma can improve the HBV infectivity (21), we decided to test whether healthy human serum (instead of plasma) contains components which can affect sHBV and ccHBV infectivity in HepG2-NTCP cells. For this purpose 5% healthy human serum (negative for HBsAg and HBsAb) was added to sHBV and ccHBV inoculums at the time of infection. Our results showed normal human serum contains components that could significantly increase the infectivity of both sHBV and ccHBV inoculums similarly, compared with the control one and all infection biomarkers significantly elevated (Figures 5A, B, C, D, E, F, G, H). The effect of adding human serum on improving ccHBV infectivity was significantly higher than in spinoculation and the suspended cell condition based on different infection markers. In addition, adding human serum to sHBV inoculum leads to significant elevation of only HBsAg and HBeAg compared with spinoculation and the suspended cell condition. Adding Myr-B to sHBV and ccHBV inoculums containing human serum significantly inhibited the infection, suggesting the presence of host factors in human serum which may facilitate HBV entry. The effect of both 5 and 10% human serum was similar and no significant differences between these concentrations were observed (data not shown).

To assess whether this effect is limited to human serum or other species' serum also contain a factor that could facilitate the HBV infection, we included monkey, duck, sheep, cow serum, and FBS to our study (final concentration of all was 5%). Our results showed that in addition to human, monkey

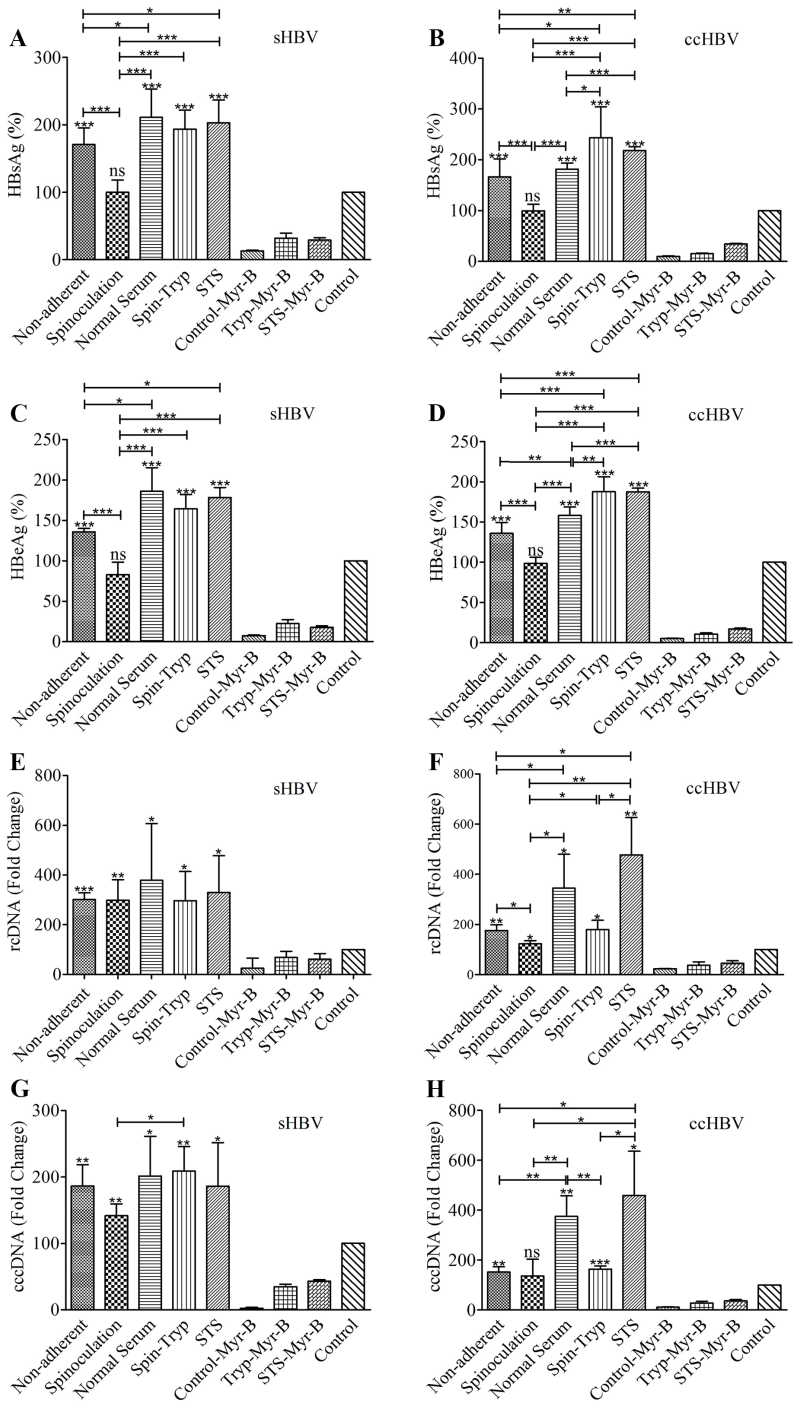

Figure 5. Effect of trypsinization, spinoculation, and addition of healthy serum on virus infectivity. HepG2-NTCP cells were cultured for 3 days to reach 80-90% confluency, then differentiated for 48h in a differentiation medium containing 1% FBS and 1.74% DMSO. On the infection day (0 dpi), differentiated HepG2-NTCP cells were infected with sHBV and ccHBV by applying different modifications individually or in combination. Infection was performed using virus inoculums containing MOI 400 and 2.5% PEG 8000 for sHBV and MOI 400 and 5% PEG 8000 for ccHBV infection. In the control group, the cells were infected with no modification. The secretion of HBsAg (A, B) and HBeAg (C, D) were detected by ELISA at 13dpi and presented as % relative to the control group. rcDNA (E, F) and cccDNA G, H) were measured by q-PCR in the lysate of infected cells and normalized to the Prnp gene, and plotted as a fold change relative to the control group. Data are shown as mean ± SD of at least three independent experiments. *P<0.5, **P<0.01, ***P<0.001, n.s: not significant, spin-tryp: spinoculation+trypsinization, STS: spinoculation+trypsinization+healthy human serum, Myr-B: Myrcludex-B, S/CO value: sample/cutoff.

serum could significantly improve ccHBV infection, while duck, sheep, cow serum and FBS did not have any significant effect (based on HBsAg and HBeAg). Therefore, we could assume that there is a certain substance in human and monkey serum which favors HBV infection in vitro (Figure 6).

Combination of suspended cells, spinoculation and healthy human serum enhances HBV infectivity. Although spinoculation and suspended conditions have been previously used to augment the HBV infection in HepG2-NTCP cells, the combination of these two modifications has not previously been applied simultaneously in HBV infection. We tested the combination effect of these parameters at the time of infection. As shown in Figures 5A, B, C and D the combined modification (spin-tryp) significantly increased HBsAg and HBeAg secretion compared with the effect of each single parameter following infection with both sHBV and ccHBV. However, no synergistic increase in HBV rcDNA or cccDNA expression level was observed (Figures 5E, F, G, H). We also evaluated the combined effect of trypsinization, spinoculation and normal serum (STS) on virus infectivity. No significant difference was observed comparing STS with the combination effect of spinoculation plus trypsinization (spin-tryp) for both sources of virus, with the exception of rcDNA and cccDNA which were significantly elevated following infection with ccHBV, but not sHBV (Figures 5E, F, G, H).

Continuous treatment of infected cells with PEG reduces HBV infection. We tested whether maintaining PEG in culture medium of ccHBV and sHBV infected cells during 13 days of infection could improve HBV infection. As shown in Figures 7B and 7C (HBsAg) and Figures 7D and 7E (HBeAg), continuous treatment of infected HepG2-NTCP cells with PEG, significantly reduced the sHBV and ccHBV infectivity. Since the levels of HBsAg and HBeAg reduced following the infection, the analysis of rcDNA and cccDNA was not conducted.

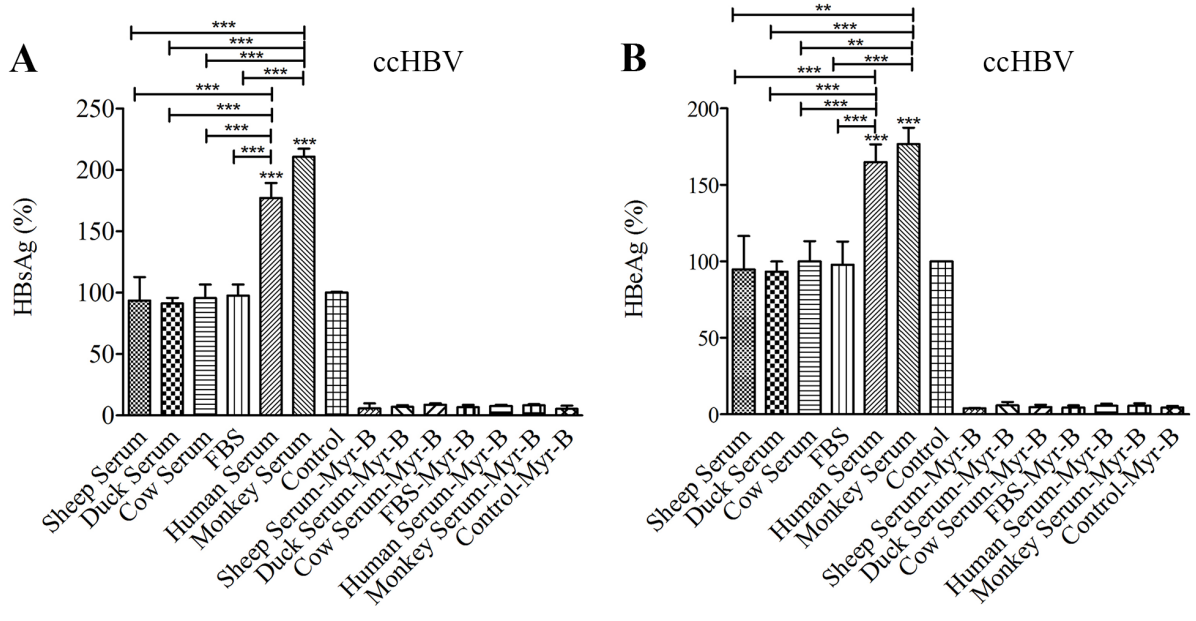

Figure 6. Human and Monkey serum significantly improve ccHBV infection in HepG2-NTCP cells. HBsAg (A) and HBeAg (B) were measured in the supernatant of HepG2-NTCP cells infected with ccHBV inoculum by ELISA at 13dpi. HepG2-NTCP cells were infected with MOI 400 of ccHBV in the presence of 5% PEG and also 5% of a healthy human, monkey, duck, sheep, cow serum, and FBS. After overnight incubation at 37°C, the wells were washed 3 times with PBS, and then the differentiation medium was added to the cells until the end of the experiment, which was 13 days (the cell culture medium was changed every 4 days). The results of HBsAg and HBeAg were presented as % relative to the control group. Data are shown as mean ± SD of three independent experiments. **P<0.01, ***P<0.001, Myr-B: Myrcludex-B, S/CO value: sample/cutoff.

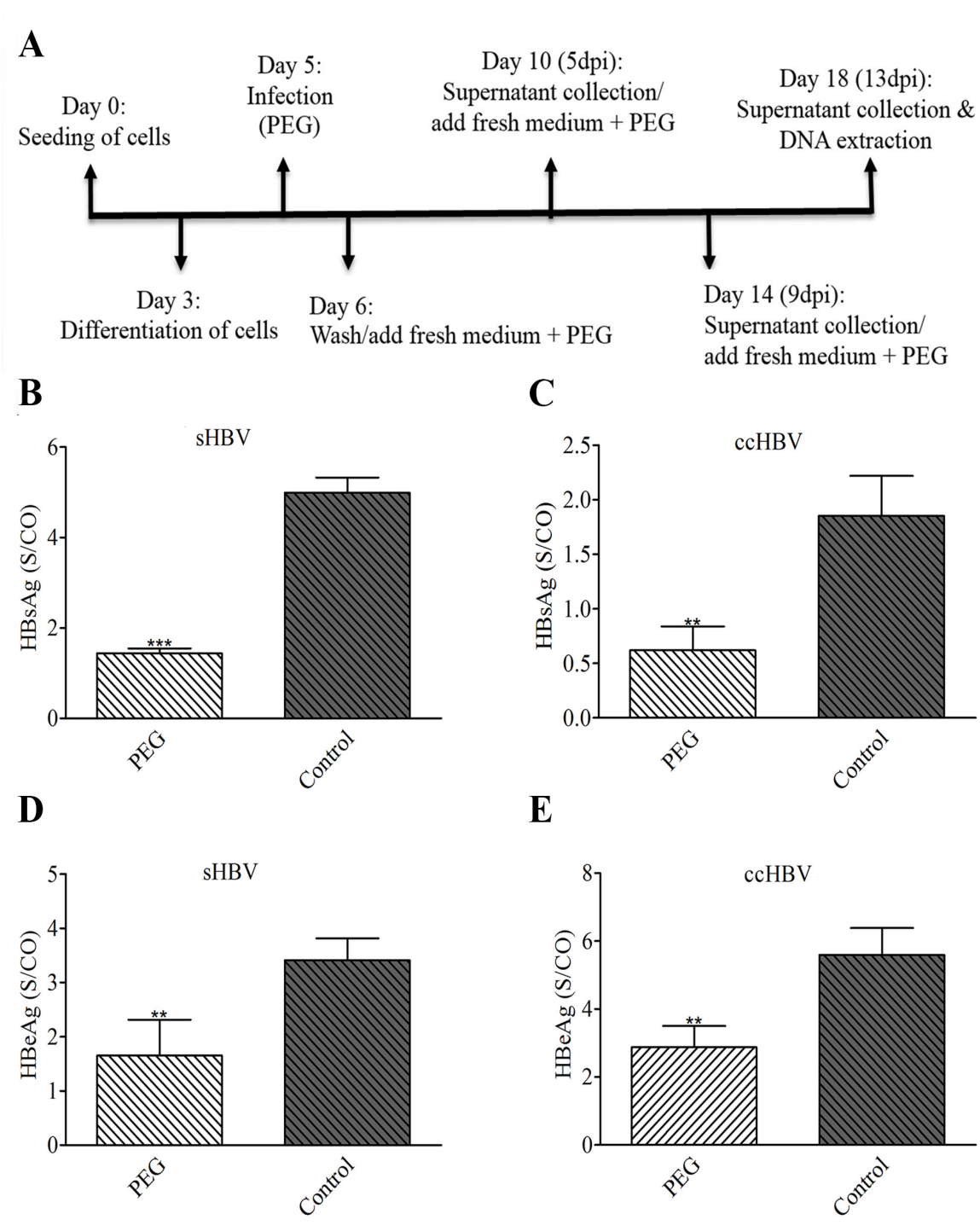

Figure 7. Continuous treatment of sHBV and ccHBV infected cells with PEG reduces HBV infection in HepG2-NTCP cells. (A) Schematic illustration of experimental design. (B, C) HBsAg and (D, E) HBeAg were measured in the supernatant of HepG2-NTCP cells infected with sHBV and ccHBV inoculums by ELISA at 13dpi. HepG2-NTCP cells in both the control and PEG groups were infected with MOI 100 of both sHBV and ccHBV in the presence of 2.5% PEG. After overnight incubation at 37°C, the wells were washed 3 times with PBS, and then the differentiation medium without PEG (the control group) or with 2.5% PEG (PEG group) was added to the cells until the end of the experiment, which was 13 days (cell culture medium was changed every 4 days). Data are shown as mean ± SD of three independent experiments. **P<0.01, ***P<0.001, S/CO value: sample/cutoff.

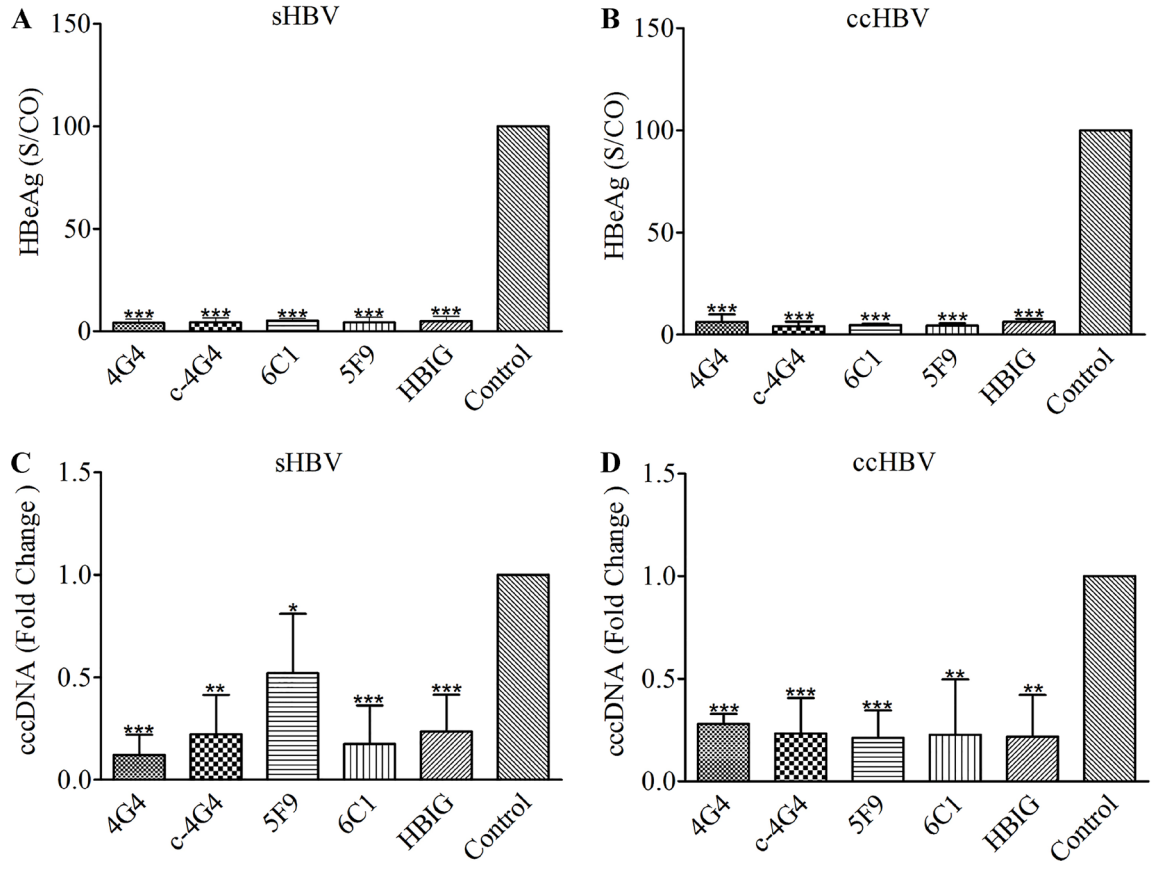

Figure 8. Neutralization of HBV infection by anti-HBs MAbs in HepG2-NTCP cells.
In this experiment, the cells were infected with no modification. mAbs at a concentration of 20ug/ml were pre-incubated for 3 h at 37°C with MOI 400 of sHBV and ccHBV and subsequently added to differentiated HepG2-NTCP. After overnight incubation, cells were washed three times with PBS and maintained in the differentiation medium until 13 days after infection. 0.1 IU/ml of commercial polyclonal anti-HBs antibody (HBIG) was used as a control. Quantification of HBeAg (A, B was performed by ELISA at 13dpi in the supernatant of sHBV and ccHBV infected cells and presented as S/CO. Quantification of cccDNA (C, D) in cell lysates of infected cells at 13 dpi was performed by q-PCR. cccDNA levels were normalized to Prnp, and were plotted as a fold change relative to the infected cells in the absence of anti-HBs MAbs. The data are shown as mean ± SD of three independent experiments. *P<0.5, **P<0.01, ***P<0.001, ns: not significant, S/CO value: sample/cutoff.

Cell viability with trypan blue at 13 dpi infection was determined, and no significant difference among PEG treated and the control group was observed (data not shown).

Assessment of HBV neutralization by anti-HBs MAbs in HepG2-NTCP cells. sHBV and ccHBV inoculums were pretreated with 20 µg/ml of three different murine anti-HBs MAbs (with 2 different isotypes) and also one chimeric human-murine anti-HBs MAb and incubated for 3 h and then added to the differentiated HepG2-NTCP cells. At 13dpi, secretion of HBeAg (Figures 8A, B) in the supernatant and expression of cccDNA (Figures 8C, D) in the lysate of cells were measured to evaluate neutralization efficacy of antibodies. Different isotypes of antibodies were included in this study to assess whether differences in isotypes of antibodies could affect the pattern of infection markers. The MAbs employed in this study were murine 4G4 and 6C1 (IgG2b), 5F9 (IgG1), and chimeric human-murine c-4G4 (IgG1) (derived from murine 4G4) (25, 26, 31). The results showed that HBeAg and cccDNA were significantly suppressed by all MAbs.

MAbs 4G4, 6C1, and 5F9 were able to almost completely inhibit both ccHBV and sHBV infection in HepG2-NTCP cells based on HBeAg results (more than 90% of inhibition). The inhibitory effect of chimeric 4G4 was similar to that of its murine counterpart, as expected. As the results of cccDNA shows (Figures 8C, D), all MAbs significantly inhibited both ccHBV and sHBV infection.

DISCUSSION

We designed a comprehensive study to investigate different parameters, previously published and novel, individually or in combination and simultaneously compared the infectivity of two sources of HBV inoculum (ccHBV and sHBV). The variables which have been tested individually or in combination include viral load (MOI), different concentrations of PEG, spinoculation, cell trypsinization and presence of human normal serum, monkey, duck, sheep, fetal and adult bovine serum.

The sHBV and ccHBV infectivity was compared using different viral loads from MOI 100 to 800. Our results showed that the infection establishment by both virus sources was dose-dependent; however, ccHBV was more infectious than sHBV according to HBeAg and rcDNA level (Figure 1). It has been previously reported that HepAD38 secreted 10-fold more non-infectious ‘‘naked” capsids than the infectious enveloped virions, while naked capsids are absent in patients serum (13). Naked capsids contain the virus DNA but they are not infectious, thereby the number of infectious viruses have been underestimated in our ccHBV inoculum. Immunoprecipitations of HBV virions from culture supernatant of HepaD38 cell line and also serum patients would give more accurate results for the comparison of these two different sources of the virus and is one of the shortcomings of our study. Although the actual number of infectious HBV virions in ccHBV have been underestimated in our study, ccHBV showed higher infectivity than sHBV.

The detection of high levels of HBsAg in culture supernatant raised questions about the reliability of HBsAg as a marker of productive sHBV infection. Our results showed that some of the HBsAg detected in supernatant of sHBV infected HepG2-NTCP cells, even at 26 dpi, is not the result of sHBV infection and other infection markers, and particularly HBeAg should be evaluated for the reliability of the results and the validation of establishment of sHBV infection. sHBV and to a lesser extent ccHBV contain high level of HBsAg due to the presence of subviral particles. In addition, the concentration of HBsAg in both inoculums was determined and the results showed that sHBV and ccHBV inoculums contained 2.9 and 0.03 mg/ml HBsAg, respectively. As our results showed, HBsAg amount in sHBV inoculum was almost 100 times more than that of ccHBV inoculum. Therefore, DNA/HBsAg in sHBV and ccHBV was 1.6×10^{9} and 2.4×10^{11}, respectively.

As the results of IF assay show, the level of HBsAg at 13dpi in sHBV infected cells is slightly higher than that in ccHBV, implying that significantly higher level of HBsAg in ELISA is not the result of the established infection and could be due to the shedding of subviral particle. While HBsAg is not a reliable marker for the detection of sHBV infection even at 13 dpi, it could be used to detect ccHBV infection at 13 dpi in the supernatant of infected cells. Since ccHBV inoculum contains less amount of HBsAg compared with sHBV, almost the entire HBsAg detected at 13 dpi in the supernatant of HepG2-NTCP cells infected with ccHBV seems to be associated with the infection established in these cells, not to the shedding of subviral particles. We used Myr-B as an HBV entry inhibitor. The level of HBsAg in the presence of this inhibitor after 13 dpi in the supernatant of cells infected with sHBV was almost 6.7 times greater than that in the supernatant of the cells infected with ccHBV (Figures 4A, B). Therefore, if sHBV infection is to be evaluated by measuring the level

of HBsAg, Myr-B or other HBV infection inhibitors must be included as a control to assure the reliability of the test. Although the level of HBsAg in the supernatant of Myr-B-sHBV infected cells was 6.7 times higher than that in Myr-B-ccHBV infection at 13 dpi, there was no noticeable difference between the patterns of infection based on measuring HBeAg in most experiments.

Even at 13 dpi when HBsAg becomes a more reliable marker in sHBV infection, the HBsAg/HBeAg ratio in sHBV infected HepG2-NTCP cells (8.6) was higher than that of ccHBV infected cells (0.3), using MOI 400 (Figure 4E). On the other hand, replicative rcDNA/cccDNA ratio in sHBV infected HepG2-NTCP cells (14.11) was much lower than that of ccHBV infected cells (55.60) at MOI 400 (Figures 1D, E), which was consistent with the fact that ccHBV was more infectious than sHBV. Based on a previous study, Genotype D HBV releases less HBsAg than genotypes A to C (28); however, since the genotype of HBV of ccHBV and sHBV sources was the same, so the low HBsAg/HBeAg ratio after ccHBV infection cannot be attributed to the difference in the genotype of HBV in the two sources of virus we used in our study. The high HBsAg/HBeAg ratio in sHBV infection is probably due to an excess of subviral particles over Dane particles (complete virions) in the serum of CHB patients that attach to HBV receptors on the HepG2-NTCP cells and release gradually after adding the viral inoculum on cells (32). Consistent with our results, another study (28) also reported that HBsAg was an unreliable infectivity marker at the early stage of sHBV infection.

Our results showed that sHBV infection does not require virus purification from viremic serum samples and directly inoculating viremic serum samples, with high copy number of HBV, to HepG2-NTCP cells induced secretion of high levels of HBeAg and HBsAg. These findings contradict recently published data which showed that sHBV (genotype C) could poorly establish infection in HepG2-NTCP cells (28). However, we showed that sHBV (genotype D) could effectively infect HepG2-NTCP cells, although the level of sHBV infection at corresponding MOI was lower than that of ccHBV. Based on that study, a low HBsAg/HBeAg ratio by ccHBV-infected HepG2/NTCP cells was attributable to HBV genotype D. However, we showed that sHBV genotype D we used in our study also showed a high HBsAg/HBeAg ratio. This report also indicated that sHBV infection in HepaRG cells required partial virus purification from viremic serum samples (28), which is inconsistent with our results. This discrepancy could be due to the use of different virus genotype (genotype C in their study versus genotype D in our study) or viral load of the sera. We employed a pooled serum collected from chronically infected Iranian patients with genotype D (33) that contained a very high HBV titer, 4.7×10^9 copy/ml. Availability of patients' sera with high HBV viral load may overcome the need for PEG precipitation and concentration of HBV. We showed that HepG2-NTCP cells were easily infected with sHBV even at MOI 100 (Figures 1B, C, D, and E); however, the infectivity of these cells with ccHBV was almost 2.7 times higher than that induced with sHBV according to HBeAg level at MOI 100 (Figure 1C). The only HBV genotype detectable among Iranian patients is genotype D. Since we did not have access to the patients' sera with other genotypes, we could not evaluate the infectivity of sHBV with genotypes other than D.

Increasing the concentration of PEG up to 5% in ccHBV inoculum improved the infection dose-dependently, while 10% PEG showed an inverse effect and diminished ccHBV infectivity. However, the effect of PEG on ccHBV infectivity was more significant than sHBV infectivity (Figure 3). Our results, and for the first time, showed that adding 5% of PEG, which is commonly used to improve in vitro infection of ccHBV, significantly reduced sHBV infection. However, this result is contradictory to a previous study which showed that cccDNA strongly increased in

the presence of increasing concentration of PEG (3, 3.5, 4, 4.5, and 5%) after the infection of PHH with sHBV (20) (the genotype of sHBV was not reported in the study). Based on our results, the optimal concentration of PEG that lead to the optimized infection in HepG2-NTCP cell is different for sHBV and ccHBV. Increasing the concentration of PEG during infection with sHBV led to higher HBsAg/HBeAg ratio (Figures 3A, B, C, D). As mentioned above, removing PEG from culture medium or adding 1.25 or 2.5% PEG during sHBV infection had no noticeable effect on HBsAg/HBeAg ratio and resulted in a similar HBsAg/HBeAg ratio in all the three conditions, whereas adding 5 or 10% PEG during sHBV inoculation caused a noticeable increase in this ratio. In fact, the inhibitory effect of 5 and 10% of PEG during sHBV infection was accompanied by high HBsAg/HBeAg ratio. The increasing HBsAg/HBeAg ratio may be due to higher aggregation of Dane particles and SVP at high concentration of PEG that can facilitate their binding to surface of cells. This has two consequences; inhibiting entry of Dane particles into cells as well as continuous shedding of trapped SVPs even at 13dpi. In addition, our results contradicted a previous study showing that maintaining PEG in culture medium until 13dpi increased the rate of infection by promoting the virus spread (17). Our results showed that maintaining PEG for 13 dpi not only did not improve the infection rate, but also significantly reduced the rate of both sHBV and ccHBV infections. Similarly, a recent publication used two different clones of HepG2-NTCP and evaluated the effect of continuous presence of PEG on virus spread. Their results showed that in one clone HBV spread happened in the absence of PEG and PEG supplementation did not affect virus spreading, while in the other clone no virus spread was detectable either at presence or absence of PEG (13). Thus, the effect of continuous PEG treatment in favor of HBV spread in HepG2-NTCP cells might be inconsistent in different clones of HepG2-NTCP cells and further publications that use different clones of HepG2-NTCP are needed to reach a conclusion.

Since the differences in clones of HepG2-NTCP can affect the pattern of HBV infection, we used only one pooled human serum and also one clone of HepG2-NTCP in all experiments to minimize the effect of different sera and also different clones of HepG2-NTCP.

The inhibitory effect of continuous treatment of cells with PEG might be due to the long term toxicity of PEG, evidenced by our data that showed a significant decreased viability of the cells (data not shown). In conclusion, although previous studies have shown the positive effect of PEG on HBV infection, our study has advantages over the previous studies in two aspects: first, it included both cell culture-derived and serum-derived HBV simultaneously, providing access to the infectivity feature of two different sources of the virus at the same time in the presence of PEG. Second, the effect of PEG on virus infection in a dose-dependent manner was simultaneously evaluated in both sources of the virus.

In addition to PEG, other variables including spinoculation and the use of suspended cells and healthy serum were tested to further optimize the HBV infection. The results showed that the use of trypsinized suspended cells, spinoculation, and the presence of human serum individually or in combination improved HBV infection, the combination did not show synergistic effect for all the infection markers (Figures 5A, B, C, D, E, F, G, H). Furthermore, the combination of all modifications (STS) significantly improved ccHBV infection compared with spinoculation and suspended cell condition and lead to a significant increase in HBsAg, HBeAg, cccDNA, and rcDNA. However, applying STS during sHBV infection lead to a significant increase of only HBsAg and HBeAg but not rcDNA and cccDNA compared with the individual modification. These results are in line with the previous

reports which showed the positive effect of spinoculation and employment of suspended cells on the rate of infection (29, 30). It has been previously shown that spinoculation improved ccHBV infection (29). Our results showed a significant increase in only rcDNA and cccDNA but not HBsAg and HBeAg after sHBV infection (Figures 5A, C, E, G). In addition, the spinoculation significantly elevated only HBV rcDNA but not the other markers after the infection with ccHBV in HepG2-NTCP cells (Figures 5B, D, F, H).

Furthermore, we observed that healthy human serum contains components which can improve the infection with both sHBV and ccHBV sources. The effect of adding human serum on improving ccHBV infectivity was significantly higher than spinoculation and suspended cell condition based on different infection markers. Consistent with our results, healthy human plasma, and due to its containing heparin, has recently been shown to facilitate HBV infection (21). Next, we decided to assess whether other species' serum contains a factor which favors HBV infection in vitro. Our results, and for the first time, showed that human and monkey serum could significantly improve ccHBV infection, while duck, sheep, cow serum and FBS did not have any significant effect. Therefore, certain soluble factors, commonly present in human and monkey serum and plasma, could apparently facilitate both sHBV and ccHBV infectivity and provide a PEG-free infection system closely resembling human natural infection. It is noteworthy that "heparin" does not seem to be the only component in serum or plasma which can improve HBV infectivity, as other species' serum also contain heparin, while adding their serum did not affect HBV infectivity. Further research is needed to determine which fraction of human and monkey serum is responsible for improving HBV infection in HepG2-NTCP.

Following the optimization of the sHBV and ccHBV infection condition in HepG2-NTCP cells, we sought to check applicability and sensitivity of this system for the assessment of HBV neutralizing agents. A number of neutralizing anti-HBs MAbs were selected for this experiment. As Figure 8 shows, these murine and chimeric MAbs were able to almost completely inhibit HBV infection, based on HBeAg and cccDNA at 13 dpi.

In conclusion, we developed a cell-culture system utilizing HepG2-NTCP cells which is permissive to both ccHBV and sHBV sources. Our results showed that in addition to human serum, monkey serum could significantly improve ccHBV infection, while fetal and adult bovine serum as well as duck and sheep serum did not have a promotive effect. In addition, we showed that the combination of spinoculation, using suspended cells and also adding human or monkey serum to HBV inoculum is an intervention that could significantly improve the permissivity of HepG2-NTCP cells to HBV infection compared with the individual strategies.

Our optimized in vitro system is applicable for the assessment the neutralizing efficacy of anti-HBs MAbs and could be a valuable tool for the analysis of other HBV neutralizing therapeutics.

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Conflict of Interest: None declared.

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