

# Dynamic correlation between induction of the expression of heme oxygenase-1 and hepatitis B viral replication

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**Abstract.** Heme oxygenase-1 (HO-1) possesses significant potential as a drug target for hepatitis B, which may be transferable to patient therapy. The aim of the present study was to clarify the dynamic correlation between the hepatitis B virus (HBV) and HO-1. The levels of HBV replication and expression of HO-1 were investigated in HepG2.2.15 hepatoma cells following exposure to 5-50  $\mu$ M hemin for 1-6 days. The mRNA expression levels of HO-1 were then detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HBV replication levels were determined by enzyme-immunoassay and a PCR-fluorescence quantitation assay. The results of the present study demonstrated that the mRNA expression levels of HO-1 increased in a dose-dependent manner in the HepG2.2.15 cells, following exposure to 5-50  $\mu$ M hemin. The mRNA expression levels of HO-1 reached a peak following exposure of the cells to hemin for three days, subsequently the expression of HO-1 decreased. Following exposure to hemin at an optimal concentration of 50  $\mu$ M for 1-6 days, the levels of the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the cells were significantly reduced. This marked reduction in the expression of HBsAg and HBeAg reached its peak on the first day, following which the inhibition weakened as the duration of exposure increased. In addition, the inhibition of HBV DNA replication increased with the a longer duration of exposure. Furthermore, HBV DNA levels were significantly decreased following exposure to hemin for 3-6 days. In conclusion, the present study demonstrated that induced expression of HO-1 interfered with HBV replication in a dose and time-dependent

manner, implying that a reduction of the HBV viral load may contribute to upregulation in the expression of HO-1.

## Introduction

Hepatitis B is the most common severe hepatic infection worldwide. The causative agent of hepatitis B is the hepatitis B virus (HBV), which attacks the liver. There is currently no complete cure for chronic hepatitis B. The drugs currently used to treat hepatitis B slow the progression of liver disease, by suppressing the virus. If less HBV is produced, less liver damage occurs (1). However, the current anti-HBV drugs, including interferon- $\alpha$  and nucleoside analogues, have certain disadvantages, including the rebound phenomenon of HBV replication following withdrawal of treatment and unpleasant side effects (2). Furthermore, a significant number of patients develop drug resistance following long-term use of these therapeutic agents (3). Therefore, further investigation is required, in order to develop novel anti-HBV agents.

Heme oxygenases (HO) are rate-limiting enzymes in heme catabolism. They catalyze the oxidative degradation of heme to carbon monoxide, free iron and biliverdin, which is subsequently converted to bilirubin by the bilirubin reductase enzyme (4,5). Among the three known heme oxygenase isoforms, HO-1, HO-2 and HO-3; HO-1 is the only inducible form present in almost every type of cell (6). The induction, overexpression or inhibition of HO-1 degradation has been observed to interfere with acute and chronic liver inflammation and fibrotic progression, apoptotic liver damage, replication of hepatitis B and C viruses or protection of liver grafts from ischemia/reperfusion injury, caused by either transplantation or hemorrhage/resuscitation (7). Therefore, HO-1 possesses significant curative potential, particularly for patients with hepatitis B and C, predominantly due to its anti-inflammatory, anti-apoptotic, antifibrotic and antiviral properties (7).

The induction of HO-1 directly represses HBV replication in hepatocytes at the post-transcriptional level by reducing the stability of the HBV core protein, therefore inhibiting the refill of nuclear HBV covalently closed circular DNA (HBV cccDNA) (8). Small interfering (si)RNA, directed against HO-1 demonstrated that this effect is dependent on the expression of HO-1 (7). Therefore, the induction of HO-1

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may be a novel therapeutic option for hepatitis B-associated inflammation. Although HO-1 has been observed to exhibit pronounced antiviral properties, the dynamic association between HBV replication and the expression of HO-1 remains to be elucidated. The present study aimed to investigate the overexpression of HO-1 induced by hemin, and to discuss the dynamic association between HBV replication and the induction of HO-1 in HBV-transfected HepG2.2.15 cells.

## Materials and methods

**Reagents.** Fetal bovine serum, hemin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Life Technologies (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A HBV DNA polymerase chain reaction (PCR)-fluorescence quantitation kit and enzyme-immunoassay (EIA) kits for the detection of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were obtained from Shanghai Kehua Bio-engineering Co., Ltd (Shanghai, China). G418 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup>, RNAiso Plus, a Prime Script Reverse Transcription (RT) Reagent kit and DNA ladder marker were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). All other reagents were of the highest commercial grade available.

**Cytotoxicity of hemin on HepG2.2.15 cells.** HBV-transfected HepG2.2.15 hepatoma cells ( $5.0 \times 10^4$  cells/ml) were provided by the State Key Laboratory for Diagnosis and Treatment of Infectious Diseases at Zhejiang University (Zhejiang, China) (9) and maintained in DMEM supplemented with 2 mM glutamine, 10% (v/v) heat-inactivated fetal bovine serum and 380  $\mu$ g/ml G418 at 37°C in a 95% humidified atmosphere containing 5% CO<sub>2</sub>. Hemin-induced cytotoxicity was analyzed using an MTT assay. Briefly, the cells were seeded in a 96-well plate ( $5.0 \times 10^4$  cells/ml; 200  $\mu$ l/well). Different concentrations of hemin (5, 10, 20, 50, 75 and 100  $\mu$ M) were applied to the culture wells in triplicate. The cells were then incubated for six days and 10  $\mu$ l MTT (5 mg/ml) was then added to the cells in each well. Following 4 h culture at 37°C (95% humidity; 5% CO<sub>2</sub>), the medium was removed, and the blue formazan crystals, which had formed, were dissolved in 150  $\mu$ l dimethyl sulfoxide (Sigma-Aldrich). The absorbance of the formazan generated from MTT was measured at a wavelength of 570 nm using a Multi-Well microplate reader (Multiskan MK3; Thermo Fisher Scientific Inc., Waltham, MA, USA). The cytotoxicity of hemin was defined according to the quantity of formazan produced relative to that produced by the untreated cells, and was expressed as cell viability.

**Hemin-induced expression of HO-1 in HepG2.2.15 cells.** HepG2.2.15 cells were seeded in six-well plates ( $5.0 \times 10^4$  cells/ml) and were treated with various concentrations of hemin (5, 10, 20 and 50  $\mu$ M) for 1-6 days, respectively. The mRNA expression of HO-1 was detected using quantitative (q)PCR. Total RNA was extracted from the HepG2.2.15 cells using RNAiso Plus reagent, followed by recombinant DNase I (RNase-free; Takara Biotechnology Co., Ltd) digestion. The RNA was reverse transcribed into cDNA using the Prime Script RT reagent kit,

according to the manufacturer's instructions. The products of reverse transcription served as a template for qPCR. qPCR was performed with SYBR Premix Ex Taq (Tli RNaseH Plus) using a Bio-Rad iQ5 Real Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).  $\beta$ -actin was used as an internal control. The primers for HO-1 and  $\beta$ -actin were specifically designed according to the mRNA sequences of human HO-1 and  $\beta$ -actin, obtained from Genbank ([http://blast.st.va.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE\\_TYPE=BlastHome](http://blast.st.va.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome)).

The following primer sequences were used: HO-1, forward 5'-TTGCCAGTGCCACCAAGTTC-3', reverse 5'-TCAGCAGCTCCTGCAACTCC-3'; and  $\beta$ -actin, forward 5'-TGGCACCAGCACAATGAA-3', and reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The thermal program comprised of an initial denaturation at 95°C for 30 sec, followed by 40 amplification cycles, each containing two steps: 95°C for 5 sec and 60°C for 1 min. The target gene fragments were analyzed using gel electrophoresis (2% agarose; Biowest SAS, Nuaille, France), to verify the specificity of the primers. To check the purity of the products, a melting curve analysis was performed after each run. Relative quantification was conducted using the cycle threshold (Ct) values, which were determined for triplicate reactions for the assessed and reference samples for each target gene, and for the internal control gene ( $\beta$ -actin). The relative expression levels were determined using the  $2^{-\Delta\Delta C_t}$  quantification method, where  $\Delta\Delta C_t = \Delta C_t(\text{target sample}) - \Delta C_t(\text{reference sample})$  (10).

**HBV replication in HepG2.2.15 cells treated with hemin.** The medium, in which the HepG2.2.15 cells treated with hemin for 1-6 days, was used to measure the HBsAg, HBeAg and HBV DNA levels. The levels of HBsAg and HBeAg in the culture supernatants were determined using EIA kits, according to the manufacturer's instructions. The absorbance was measured at 450 nm using a multi-well plate reader. The HBV viral load in the culture supernatants was detected using a HBV DNA PCR-fluorescence quantitation kit. Briefly, viral DNA from HBV virions present in the cell culture supernatants was extracted and amplified using the Bio-Rad iQ5 Real Time PCR system. The primer sequences were as follows: Forward 5'-CCGTCTGTGCCTTCTCATCTG-3', and reverse 5'-AGTCCAAGAGTACTCTTATAGAAGACCTT-3'. The Taqman probe sequence was: FAM-CCGTGTGCACTTCGCTTCACCTCTGC. The thermal program comprised of an initial denaturation at 94°C for 2 min, followed by 40 amplification cycles each containing two steps: 95°C for 5 sec and 60°C for 30 sec. A plasmid containing the HBV genome was used to prepare the standard curve.

**Dynamic correlation between HO-1 induction and HBV replication.** Preliminary investigations indicated that 50  $\mu$ M hemin is the maximum non-toxic concentration on HepG2.2.15 cells, and is the optimal concentration for the induction of HO-1 (Table I). To further determine the dynamic correlation between the induction of HO-1 and the replication of HBV, overexpression of HO-1 was induced using 50  $\mu$ M hemin, and its effects on HBV replication were investigated in the HepG2.2.15 cells. HepG2.2.15 cells were treated with 50  $\mu$ M hemin for 1-6 days. The expression levels of HO-1 in the cells treated with hemin for different durations were determined

Table I. Cytotoxicity of hemin in HepG2.2.15 cells.

| Group   | Hemin concentration ( $\mu$ M) | Absorbance at 570 nm           | Cell survival (%) |
|---------|--------------------------------|--------------------------------|-------------------|
| Vehicle | -                              | 1.586 $\pm$ 0.058              | 100.000           |
| Hemin   | 5                              | 1.675 $\pm$ 0.042              | 105.612           |
|         | 10                             | 1.535 $\pm$ 0.112              | 96.784            |
|         | 20                             | 1.435 $\pm$ 0.103              | 90.479            |
|         | 50                             | 1.446 $\pm$ 0.101              | 91.173            |
|         | 75                             | 1.377 $\pm$ 0.147 <sup>a</sup> | 86.822            |
|         | 100                            | 1.259 $\pm$ 0.055 <sup>b</sup> | 79.382            |

Cells were treated with hemin at various concentrations (5-100  $\mu$ M) for six days. The cytotoxicity of hemin in the cells was determined using an MTT assay. All determinations were replicated four times and values are expressed as the mean  $\pm$  standard deviation. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01, compared with the vehicle control. 0.1% ammonia water was used as the vehicle control.

using qPCR, as described above. The HBsAg and HBeAg levels in the culture supernatants were determined using EIA kits. The HBV viral load in culture supernatants were detected using a HBV DNA PCR-fluorescence quantitation kit.

**Statistical analysis.** The experimental data are expressed as the mean  $\pm$  standard deviation. The results were subjected to one-way analysis of variance and Student's t-test in order to determine significance. Statistical analyses were performed using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Cytotoxicity analysis of hemin.** The cytotoxicity of hemin on HepG2.2.15 cells was determined using an MTT assay. Hemin was non-toxic to the cells when used at a concentration between 5 and 50  $\mu$ M for six days (Table I). Following exposure to 50  $\mu$ M hemin for six days, cell viability was 91.17%. The maximum non-toxic concentration of hemin on HepG2.2.15 cells was 50  $\mu$ M, whereas treatment with hemin at concentrations between 75 and 100  $\mu$ M produced a significant decrease in cell viability. Cell viability was 86.82 and 79.38% following exposure to 75 and 100  $\mu$ M hemin for six days, respectively.

**Hemin induces the expression of HO-1.** The mRNA expression levels of hemin-induced HO-1 were determined in the HepG2.2.15 cells by qPCR (Fig. 1). Treatment with hemin at 5-50  $\mu$ M significantly increased the expression levels of HO-1 in a dose-dependent manner. Exposure of the cells to hemin for different durations resulted in an elevation in the mRNA expression levels of HO-1 (Fig. 2). Furthermore, the mRNA expression of HO-1 peaked following exposure to 5-50  $\mu$ M hemin for three days, whereas the mRNA expression levels began to decrease as the duration of exposure to hemin increased further.

**Effects of hemin on HBV replication.** The levels of HBsAg and HBeAg were detected in the HepG2.2.15 cell culture supernatants following incubation with hemin (5-50  $\mu$ M) for 1-6 days. The expression of HBsAg was significantly inhibited in the HepG2.2.15 cells following exposure to 5-50  $\mu$ M hemin (Fig. 3). In addition, the expression of HBeAg was markedly reduced

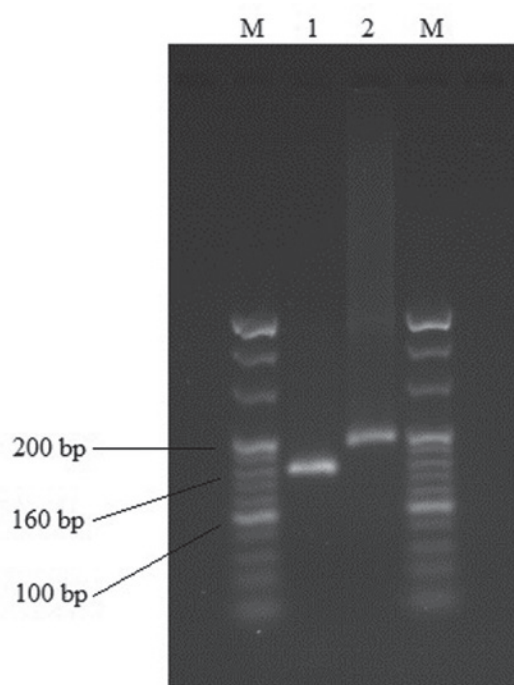


Figure 1. Polymerase chain reaction amplification products of HO-1 and  $\beta$ -actin. Lane M, DNA marker (molecular weight standard); lane 1, HO-1; lane 2,  $\beta$ -actin.  $\beta$ -actin was used as an internal control. HO-1, heme oxygenase-1; Bp, base pairs.

in the cells following exposure to 5-50  $\mu$ M hemin (Fig. 4). Furthermore, as the duration of exposure to hemin, increased, the inhibitory trend was less marked. The DNA expression levels of HBV were determined in the culture supernatants following treatment of the cells with hemin (5-50  $\mu$ M) for 1-6 days. HBV DNA replication was markedly inhibited following exposure to hemin, and this trend of inhibition became more marked as the duration of exposure increased (Fig. 5).

**Correlation between the expression of HO-1 and HBV replication.** To further analyze the dynamic association between the induction of HO-1 and HBV replication, the expression of HO-1 was induced in HepG2.2.15 cells with hemin, at an optimal concentration of 50  $\mu$ M, for 1-6 days (Table II). The

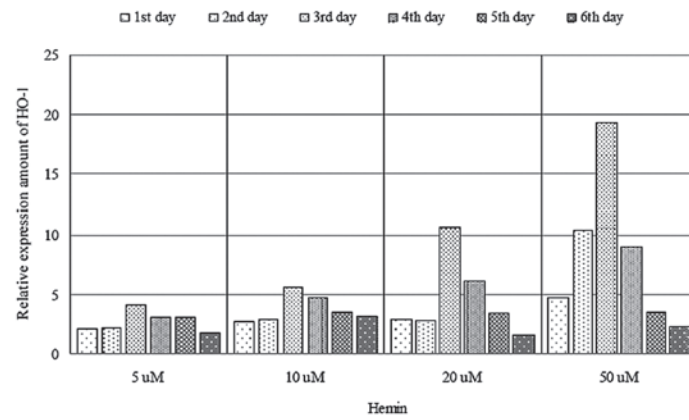


Figure 2. Relative expression levels of HO-1 induced by hemin in HepG2.2.15 hepatoma cells. The cells were treated with hemin at concentrations between 5 and 50  $\mu$ M for 1-6 days.  $\beta$ -actin was used as an internal control. 0.1% ammonia water was used as the vehicle control. HO-1, heme oxygenase-1.

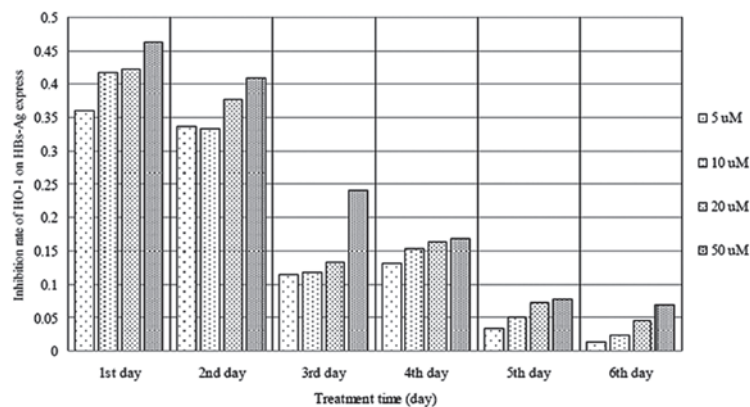


Figure 3. Effects of hemin-induced expression of HO-1 on the expression of HBsAg in HepG2.2.15 cells. The cells were treated with hemin at concentrations between 5 and 50  $\mu$ M for 1-6 days. 0.1% ammonia water was used as the vehicle control. HO-1, heme oxygenase-1; HBsAg, hepatitis B surface antigen.

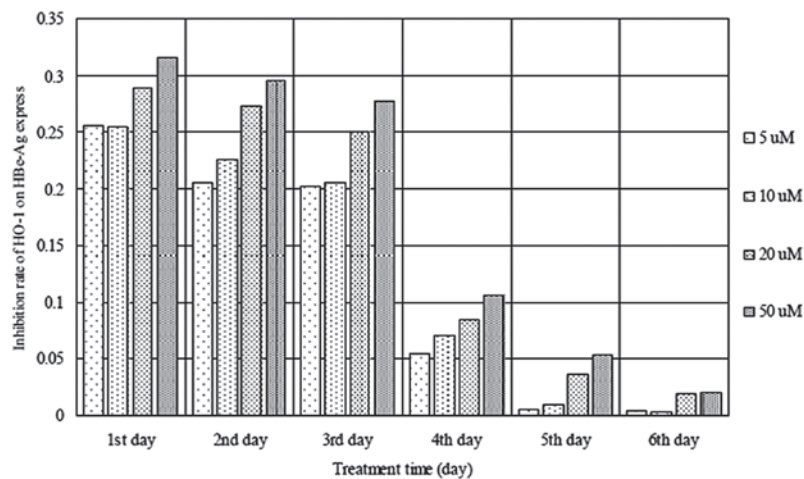


Figure 4. Effects of hemin-induced expression of HO-1 on the expression of HBeAg in HepG2.2.15 cells. The cells were treated with hemin at concentrations between 5 and 50  $\mu$ M for 1-6 days. 0.1% ammonia water was used as the vehicle control. HO-1, heme oxygenase-1; HBeAg, hepatitis B e antigen.

expression of HO-1 increased markedly following exposure of the cells to hemin for 1-6 days. The mRNA expression levels of HO-1 reached a peak in the hepatoma cells on the third day and subsequently reduced as the duration of exposure to hemin

increased. Furthermore, the levels of HBsAg and HBeAg in the culture supernatants were significantly reduced following exposure to hemin for 1-6 days. The maximum inhibition of HBsAg and HBeAg was 37.37 and 31.73% on the first day,



Table II. Effects of hemin-induced expression of HO-1 on HBV replication in HepG2.2.15 cells.

| Group              | Treatment duration (days) | Relative HO-1 expression | HBsAg                          |                     | HBeAg                          |                     | log <sup>HBV-DNA</sup> (copy/ $\mu$ l) |
|--------------------|---------------------------|--------------------------|--------------------------------|---------------------|--------------------------------|---------------------|--|
|                    |                           |                          | Absorbance                     | Inhibitory rate (%) | Absorbance                     | Inhibitory rate (%) |  |
| Vehicle            | 1                         | -                        | 0.669 $\pm$ 0.033              | -                   | 0.562 $\pm$ 0.038              | -                   | 4.842 $\pm$ 0.045                      |
|                    | 2                         | -                        | 0.791 $\pm$ 0.030              | -                   | 0.684 $\pm$ 0.064              | -                   | 4.843 $\pm$ 0.016                      |
|                    | 3                         | -                        | 1.071 $\pm$ 0.034              | -                   | 1.003 $\pm$ 0.056              | -                   | 4.822 $\pm$ 0.035                      |
|                    | 4                         | -                        | 1.524 $\pm$ 0.043              | -                   | 1.890 $\pm$ 0.108              | -                   | 4.722 $\pm$ 0.022                      |
|                    | 5                         | -                        | 2.174 $\pm$ 0.028              | -                   | 2.020 $\pm$ 0.063              | -                   | 4.772 $\pm$ 0.008                      |
|                    | 6                         | -                        | 2.367 $\pm$ 0.110              | -                   | 2.272 $\pm$ 0.075              | -                   | 4.878 $\pm$ 0.035                      |
| Hemin (50 $\mu$ M) | 1                         | 4.837 $\pm$ 0.798        | 0.413 $\pm$ 0.047 <sup>b</sup> | 37.374              | 0.383 $\pm$ 0.027 <sup>b</sup> | 31.729              | 4.771 $\pm$ 0.033                      |
|                    | 2                         | 5.807 $\pm$ 0.740        | 0.502 $\pm$ 0.027 <sup>c</sup> | 36.498              | 0.517 $\pm$ 0.023 <sup>a</sup> | 24.415              | 4.772 $\pm$ 0.049                      |
|                    | 3                         | 17.923 $\pm$ 3.061       | 0.815 $\pm$ 0.024 <sup>c</sup> | 23.832              | 0.791 $\pm$ 0.069 <sup>a</sup> | 20.900              | 4.687 $\pm$ 0.061 <sup>a</sup>         |
|                    | 4                         | 13.943 $\pm$ 0.777       | 1.183 $\pm$ 0.088 <sup>b</sup> | 22.193              | 1.618 $\pm$ 0.091 <sup>a</sup> | 14.392              | 4.595 $\pm$ 0.055 <sup>a</sup>         |
|                    | 5                         | 5.614 $\pm$ 1.146        | 1.901 $\pm$ 0.073 <sup>b</sup> | 12.412              | 1.727 $\pm$ 0.106 <sup>a</sup> | 14.505              | 4.602 $\pm$ 0.065 <sup>a</sup>         |
|                    | 6                         | 5.014 $\pm$ 0.944        | 2.112 $\pm$ 0.084 <sup>a</sup> | 10.494              | 2.093 $\pm$ 0.048 <sup>a</sup> | 7.783               | 4.639 $\pm$ 0.094 <sup>a</sup>         |

HO-1 expression levels were detected by quantitative PCR. The levels of HBsAg and HBeAg were determined by enzyme-immunoassays. The DNA expression levels of HBV were detected using a fluorescence quantitation PCR assay. The relative expression of HO-1, absorbance at 450 nm and log<sup>HBV-DNA</sup> values are expressed as the mean  $\pm$  standard deviation of three replicates. Inhibition (%) = (mean negative control absorbance - mean experimental absorbance) / (mean negative control absorbance)  $\times$  100%.  $\beta$ -actin was used as an internal control. 0.1% ammonia water was used as the vehicle control. <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001, compared with the vehicle control. HO-1, heme oxygenase 1; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; PCR, polymerase chain reaction.

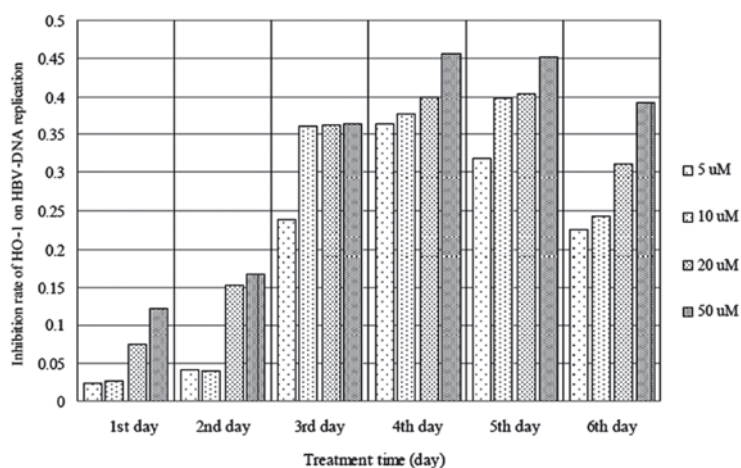


Figure 5. Effects of hemin-induced expression of HO-1 on HBV DNA replication in HepG2.2.15 cells. The cells were treated with hemin at concentrations between 5 and 50  $\mu$ M for 1-6 days. 0.1% ammonia water was used as the vehicle control. HO-1, heme oxygenase-1; HBV, hepatitis B virus.

respectively; whereas inhibition of the expression levels of HBsAg and HBeAg decreased with increasing duration of exposure to hemin. By contrast, as the duration of hemin exposure increased, the inhibition of HBV DNA replication increased. Furthermore, the expression levels of HBV DNA were markedly reduced following exposure to hemin for 3-6 days, and reached their lowest levels on the fourth day.

## Discussion

The HO system includes the heme catabolic pathway, which is comprised of HO and biliverdin reductase and the products of

heme degradation, carbon monoxide, iron and biliverdin/bilirubin (11,12). As a protective enzyme, the relevance of HO-1 in humans and animals has been demonstrated in the case of HO-1 deficiency, which results in severe tissue damage due to oxidative stress and in multiple consecutive disorders (13-15). HO-1 and the reaction products of heme degradation have been associated with cytoprotection, due to their anti-inflammatory, antioxidative, and anti-apoptotic effects (8,16). Furthermore, the induction of HO-1 or application of its products has been observed to lead to a broad range of hepatoprotective effects in animal models of acute and chronic hepatitis (7). The effects of HO-1 include preventing and attenuating liver injury (17-22),

protecting from liver fibrosis (23), interrupting the progression of nutritional steatohepatitis (24,25), preserving hepatic architecture (26), improving liver function (26,27) and providing protection against severe ischemia/reperfusion injury (27-30). Therefore, HO-1 offers a promising novel target in drug development, and the implementation of clinical approaches to moderate and alleviate the numerous chronic disorders affected by perturbations in the HO system (31).

It has been demonstrated that the induction or overexpression of HO-1 interfered with the viral replication of hepatitis B and C (8,32-35). HO-1 induction results in a marked antiviral effect in the adenoviral transfer of the HBV 1.3 genome into wild type mice and HBV transgenic animal models of acute and chronic HBV infection, respectively (8). Investigation into the effects of HO-1 on HBV replication in hepatoma cells stably transfected with HBV have revealed that induction of HO-1 directly suppressed HBV replication in hepatocytes at the posttranscriptional level by reducing the stability of the HBV core protein, inhibiting the refill of nuclear HBV cccDNA (8). Furthermore, it has been reported that microRNA-122, which is abundantly expressed in the human liver, interferes with the expression of HO-1, thereby promoting HBV replication (32,36). Conversely, HO-1 is overexpressed in various types of tumor, including hepatocellular carcinoma, and inhibiting the expression or activity of HO-1 promotes the apoptosis of cancer cells (37-39). Although its anti-apoptotic property risks the promotion of tumor growth (7), the induction of HO-1 may become a useful adjuvant for the clinical therapy of viral hepatitis.

HepG2.2.15 cells were initially derived from the HepG2 human hepatoblastoma cells, which were transfected with a plasmid containing HBV DNA. These transfected cells can stably secrete viral particles into culture medium (40). This *in vitro* system can be used to investigate the life cycle of HBV, and the reaction of immunocompetent cells to those containing HBV DNA (9). The present study investigated the dynamic correlation between the expression of HO-1 and HBV replication in HepG2.2.15 cells following exposure to various concentrations of hemin for 1-6 days. The initial comparative investigation of HO-1 expression levels following exposure to different concentrations of hemin demonstrated that hemin (5-50  $\mu$ M) significantly induced the expression of HO-1. The mRNA expression levels of HO-1 increased in a dose-dependent manner and reached a peak following exposure to hemin for 3 days. However, the expression levels of HO-1 decreased with further extension of the duration of exposure to hemin, possibly due to the functions of HO-1 as a stress-responsive enzyme (41). The present study also investigated the levels of HBsAg and HBeAg following exposure to various concentrations of hemin. The results demonstrated that the expression levels of HBsAg and HBeAg were markedly inhibited following exposure to hemin for 1-6 days. Measurement of expression of HBV DNA following exposure to various concentrations of hemin demonstrated that HBV DNA replication was significantly inhibited in a time-dependent manner. These data suggested that the secretion of HBsAg/HBeAg and HBV DNA replication were inhibited with increasing expression levels of HO-1. Furthermore, as the duration of hemin exposure increased the inhibition of HBV DNA replication was more marked, implying that the reduction of HBV replication may contribute to the induction of the HO-1 gene.

To further understand the correlation between the induction of HO-1 and HBV replication, the gene expression of HO-1 was induced in HepG2.2.15 cells by hemin at the optimal concentration of 50  $\mu$ M for 1-6 days. The significant elevation in the mRNA expression of HO-1 peaked on the fourth day, and subsequently reduced with further extension of the duration of exposure to hemin. Furthermore, the HBsAg and HBeAg levels in the cell culture supernatants were significantly reduced following exposure to hemin for 1-6 days. This marked reduction in the expression of HBsAg and HBeAg peaked on the first day, and the inhibition was weakened as the duration of exposure to hemin increased. Conversely, the inhibition of HBV DNA replication increased with increasing duration of exposure. Furthermore, DNA expression levels of HBV were significantly decreased following exposure to hemin for 3-6 days. The effects of hemin-induced HO-1 on HBsAg and HBeAg were different from those on HBV DNA. This may be associated with a non-significant correlation between either HBsAg or HBeAg levels, and HBV DNA levels (40,42). The present study demonstrated that the marked upregulation in the expression of HO-1 following treatment with hemin attenuated HBV replication in the HepG2.2.15 cells, indicating that pharmacologically inducing overexpression of HO-1 may provide protection against HBV infection. Furthermore, the downregulation of HBV replication in the cells may be associated with the reaction products of heme degradation and the inhibition of HBV reverse transcriptase (RT)- $\epsilon$  RNA interaction by hemin (7,43). HBV-RT has numerous roles in the viral life cycle and is an essential target for the development of anti-HBV chemotherapy; hemin was found to block RT interactions with viral RNA packaging signal/origin of replication  $\epsilon$ , therefore suppressing the protein-priming reaction (43). Although the induction or overexpression of HO-1 may assist as an adjuvant for clinical therapy, no specific HO-1 inducers are currently available, despite attempts, including the use of miR-196 or antagomiR-122, direct silencing of the HO-1 transcriptional repressor Bach-1 by siRNA and the use of HO-1 coupled to a protein transduction domain (7). HO-1 and its products possess a significant curative potential that may assist in the treatment of patients with hepatitis.

In conclusion, the present study clarified the dynamic correlation between the hemin-induced expression of HO-1 and HBV replication in HepG2.2.15 cells. The upregulation of HO-1 interfered with HBV replication in a dose- and time-dependent manner. The secretion of HBsAg and HBeAg, and HBV DNA replication were inhibited with induction of the expression of HO-1, suggesting that a reduction in the HBV viral load may be contributed to by overexpression of HO-1.

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