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# Altered serum copper homeostasis suggests higher oxidative stress and lower antioxidant capability in patients with chronic hepatitis B

Yansong Huang, MD[a], Yuan Zhang, MD[b], Zhexuan Lin, PhD[b], Ming Han, MD[a,*], Hongqiu Cheng, PhD[a,*]

## Abstract

Copper homeostasis can be altered by inflammation. This study aimed to investigate the alteration of serum copper homeostasis and to explore its clinical significance in patients with chronic hepatitis B (CHB).

Thirty-two patients with CHB and 10 aged- and sex-matched healthy controls were recruited. Analyses included serum levels of total copper (TCu), copper ions (Cu^{+}), small molecule copper (SMC), ceruloplasmin (CP), Cu/Zn superoxide dismutase 1 (SOD1), urinary copper, and the activities of serum CP and SOD1.

The serum TCu and urinary copper levels in patients with CHB were significantly higher than the controls ($P = .04$ and $.003$), while the serum Cu^{+} was lower than the controls ($P = .0002$). CP and SOD1 activities in the serum were significantly lower in patients with CHB compared to controls ($P = .005$) despite higher serum concentrations. In addition, serum alanine aminotransferase inversely correlated with serum CP activity ($P = .0318$, $r = -0.4065$).

Serum copper homeostasis was altered in this cohort of patients with CHB. The results suggest increased oxidative stress and impaired antioxidant capability in patients with CHB, in addition to necroinflammation. These results may provide novel insights into the diagnosis and treatment of patients with CHB.

**Abbreviations:** ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CHB = chronic hepatitis B, CP = ceruloplasmin, Cu^{+} = copper ions, GFAAS = graphite furnace atomic absorption spectrometry, HBV = hepatitis B virus, ROS = reactive oxygen species, SMC = small molecule copper, SOD1 = superoxide dismutase 1, TBIL = total bilirubin, TCu = total copper.

**Keywords:** ceruloplasmin, chronic hepatitis B, copper homeostasis, Cu/Zn superoxide dismutase

## 1. Introduction

Chronic hepatitis B virus (HBV) infection is a major cause of liver disease in many parts of the world, and it significantly increases risk for development of hepatocellular carcinoma.[1] Clinical outcomes of HBV infection are largely determined by the host immune response.[2,3] Trace elements participate in metabolism and maintain homeostasis of physiologic processes,[4] such as protein synthesis, pregnancies, and immune function.[5,6]

[a] Second Affiliated Hospital, [b] Bio-analytical Laboratory, Shantou University Medical College, Shantou, China.

* Correspondence: Hongqiu Cheng, and Ming Han, Second Affiliated Hospital of Shantou University Medical College, Dongxia North Road (Zhu-Xia Block), Shantou, Guangdong Province 515041, China
(e-mails: chenghongqiu@sohu.com, mhan@stu.edu.cn).

Copper (Cu) is a trace element that is required for synthesis of phospholipids and hemoglobin, as well as for iron absorption.[7,8] Altered serum Cu concentrations have been reported in a variety of liver diseases, including hepatocyte degeneration, hepatocellular carcinoma, and viral hepatitis.[9]

Hepatic superoxide dismutase 1 (SOD1) and ceruloplasmin (CP), which contain Cu as a key cofactor, are important antioxidant enzymes responsible for scavenging superoxide anions, a precursor of all reactive oxygen species (ROS). Thus, they protect hepatocytes from peroxidative damage.[10] However, the relationship between the serum Cu levels and the corresponding enzymatic activities in patients with chronic hepatitis B (CHB) has not been reported. This study aimed to investigate the alteration of serum Cu homeostasis and to explore its clinical significance in patients with CHB. Since the biologic activities of Cu depend on its chemical form, detection of total Cu (TCu) is inadequate for a clinical diagnosis or the study of disease mechanism. Therefore, analyses of Cu species in serum, including Cu ions (Cu^{+}) and small molecule Cu (SMC, which is loosely bound to small molecules <25 kDa) were also included in this study.

## 2. Methods

### 2.1. Study participants

This study was performed at the Second Affiliated Hospital of Shantou University Medical College, and the protocol was approved by the ethics committee of Shantou University Medical College.

Thirty-two patients with CHB and 10 age- and sex-matched healthy controls were enrolled in this study from the Second

Affiliated Hospital of Shantou University Medical College. CHB was diagnosed following the 2015 guidelines for prevention and treatment of CHB.[11] Patients with acquired immunodeficiency syndrome, history of chemotherapy, or other chronic immune disorders were excluded. Written informed consent was obtained from all patients.

Peripheral blood samples of participants were collected upon admission. Urine samples were received the first day following admission. Liver biochemistry, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and albumin (ALB) was determined using the serum samples, and the remainder was stored at −80°C until analysis of various Cu forms.

### 2.2. Detection of serum TCu, SMC, and Cu$^+$ levels

The TCu in serum and urine were measured by graphite furnace atomic absorption spectrometry (GFAAS) at 1/5 and 1/1000 dilutions, respectively, as previously reported.[12]

The level of serum SMC was determined by ultrasonic oscillation dialysis. Briefly, each dialysis device that analyzed a sample (2 mL) was made using a 1 mL sample cell and a 5 mL Eppendorf tube, with the bottom removed and sealed using a 25-kDa dialysis membrane. First, serum (200 μL) was dispensed into the inner tube, and phosphate-buffered saline (500 μL of 100 mmol/L) was added to the outer tube. Next, the dialysis device was relocated to an ultrasonic cleaner (Automatic Science Instrument Co, Ltd, Oakville, Ontario, Canada) and subjected to ultrasonic oscillation for 30 minutes. Lastly, the dialysate in the outer tube was collected for Cu determination by GFAAS.[13]

Serum Cu$^+$ samples were extracted using N-amyl alcohol, and measured by GFAAS after the precipitation of proteins with 20% trichloroacetic acid. Cu concentration was determined by AA-7000 atomic absorption spectrometer (Shimadzu Corporation, Kyoto, Japan) with a GFA-7000A graphite furnace, and tritium lamp background correction system. All measurements were carried out with Shimadzu hollow cathode lamps and pyrolitically coated graphite tubes. The furnace parameters are described in Table 1.

### 2.3. Determination of serum CP concentration and activity

Serum CP concentration and activity were determined by scattering immunoturbidimetric assay and a CP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively.

### 2.4. Determination of serum SOD1 concentration and activity

The SOD1 concentration was measured with a quantitative enzyme immunoassay (BMS2222, eBioscience, California,

America), and SOD1 activity was determined using a WST-8 assay kit (S0103, Beyotime, Haimen city, China).

### 2.5. Statistical analysis

Variables are presented as mean ± standard deviation. The normality of variables was evaluated by the Kolmogorov–Smirnov test. Comparison between groups was performed using an unpaired $t$ test or Mann–Whitney $U$ test for non-normal distribution. The associations were analyzed by Spearman correlation. All statistical analyses were carried out with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA).

## 3. Results

### 3.1. Liver biochemistry

The concentrations of serum ALT, AST, and TBIL were significantly higher in patients with CHB compared to the healthy controls (Table 2).

### 3.2. Alterations of serum and urinary Cu levels

The TCu concentration in serum was 1012.7 ± 337.0 μg/L in patients with CHB and 788.2 ± 146.4 μg/L in controls (P = .04; Fig. 1). The urinary TCu concentration was 23.6 ± 15.8 μg/L and 10.9 ± 5.8 μg/L in patients and controls, respectively (P = .003; Fig. 2). Thus, serum and urinary TCu levels in patients with CHB were significantly higher than the healthy controls. The mean serum Cu$^+$ concentration in patients with CHB (294.8 ± 117.8 μ

**Table 1**

Furnace programs for copper measurement by graphite furnace atomic absorption spectrometry.

| Temperature, °C | Time, s | Airflow, L/min |
|---|---|---|
| 60 | 3 | 0.1 |
| 120 | 20 | 0.1 |
| 250 | 10 | 0.1 |
| 400 | 10 | 1.0 |
| 400 | 10 | 1.0 |
| 400 | 3 | 0.0 |
| 1800 | 3 | 0.0 |
| 2500 | 2 | 1.0 |

**Table 2**

Demographic and clinical characteristics of patients.

| | CHB patients | Healthy controls | P |
|---|---|---|---|
| Age, y | 42.1 ± 13.1 | 37.9 ± 7.43 | .4690 |
| ALT, U/L | 835 ± 741 | 17.0 ± 8.78 | .0001 |
| AST, U/L | 506 ± 441 | 18.9 ± 4.06 | .0001 |
| TBIL, μmol/L | 130 ± 135 | 11.1 ± 2.91 | .0007 |
| ALB, g/L | 38.6 ± 4.58 | 45.5 ± 2.25 | .0003 |

ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CHB = chronic hepatitis B, TBIL = total bilirubin.
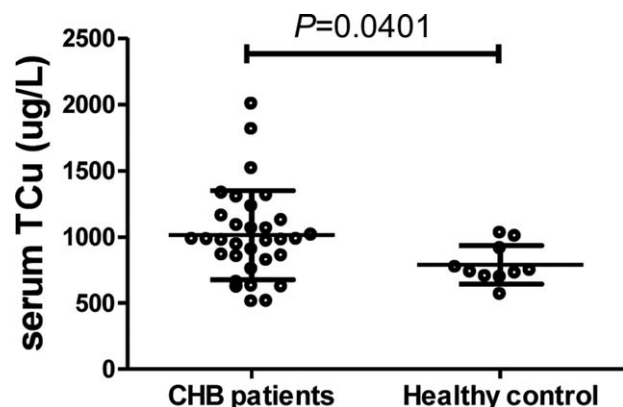


**Figure 1.** Serum total copper (TCu) concentration in patients with chronic hepatitis B (CHB) and healthy controls measured by graphite furnace atomic absorption spectrometry. Circles and the transverse lines represented the concentrations of serum TCu and quartiles of the data, respectively. The difference between 2 groups was evaluated by P value (unpaired $t$ test or Mann–Whitney $U$ test).
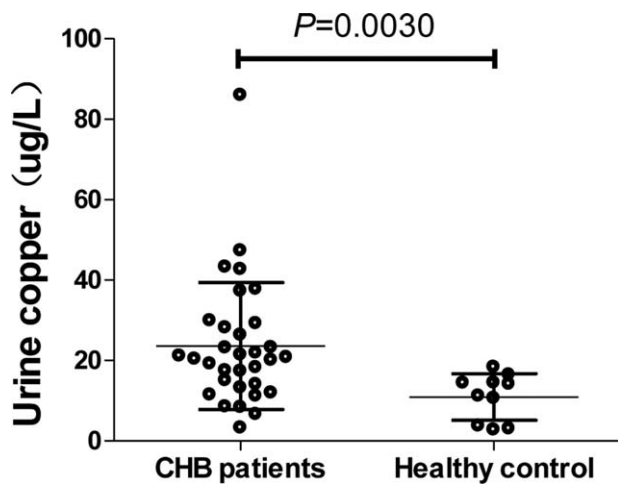
**Figure 2.** Urinary total copper concentration in patients with chronic hepatitis B (CHB) and healthy controls measured by graphite furnace atomic absorption spectrometry.



**Figure 4.** Serum ceruloplasmin (CP) concentration in patients with chronic hepatitis B (CHB) and healthy controls measured by scattering immunoturbidimetric assay.

g/L) was significantly lower than the controls ($498.2 \pm 158.7 \mu g/L$, $P < .001$; Fig. 3). No significant difference was found in serum SMC concentration between patients with CHB and healthy controls ($11.30 \pm 6.16 \mu g/L$ in patients with CHB and $10.96 \pm 4.44 \mu g/L$ in controls, $P = .68$).

### 3.3. Serum CP concentration and activity

Serum CP concentration in patients with CHB ($32.1 \pm 17.7 \mu g/L$) was higher compared to healthy controls ($20.7 \pm 3.4 \mu g/L$, $P = .015$; Fig. 4). However, serum CP activity in patients with CHB ($24.34 \pm 18.72$ U/L) was lower than the controls ($37.40 \pm 10.13$ U/L, $P = .005$; Fig. 5). In addition, the serum ALT level was inversely correlated with the serum CP activity ($P = .0318$, $r = 0.4065$; Fig. 6).

### 3.4. Serum SOD1 concentration and activity

Serum SOD1 concentration in patients with CHB ($321.0 \pm 149.9 \mu g/L$) was significantly higher than the healthy controls

($164.1 \pm 67.5 \mu g/L$, $P = .001$; Fig. 7). However, serum SOD1 activity in patients with CHB was lower compared to controls ($1.842 \pm 3.858$ U/L vs $4.01 \pm 2.48$ U/L, $P = .0039$; Fig. 8).

## 4. Discussion

In the present study, we determined serum Cu homeostasis indices including the concentration of serum TCu, SMC, and Cu⁺, urinary TCu, as well as the concentration and activity of CP and SOD1 in a group of patients with CHB. We found that both serum and urinary TCu levels were elevated while the serum Cu⁺ level in patients with CHB was reduced compared to healthy controls. Furthermore, we noted that although the serum concentration of CP and SOD1 were increased, the activity of CP and SOD1 were actually decreased in patients with CHB compared to the control group.
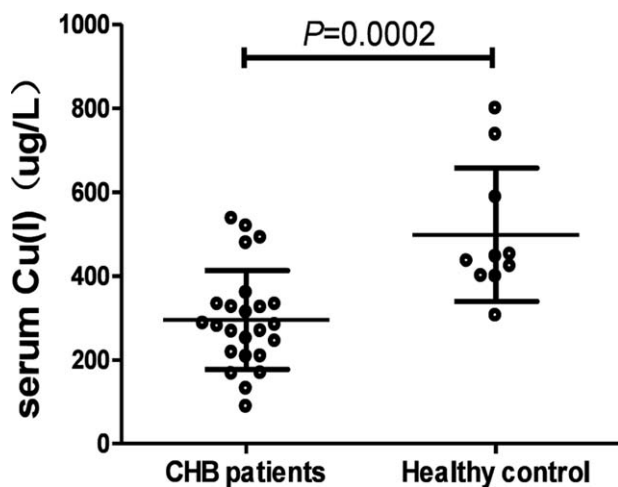


**Figure 3.** Serum Cu⁺ concentration in patients with chronic hepatitis B (CHB) and healthy controls measured by graphite furnace atomic absorption spectrometry after extraction.
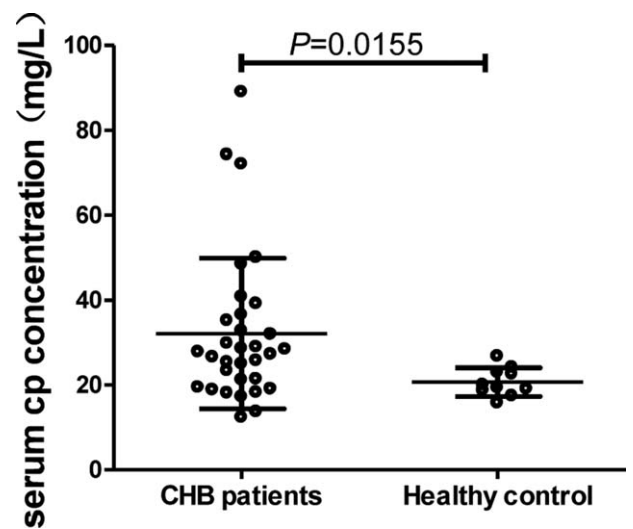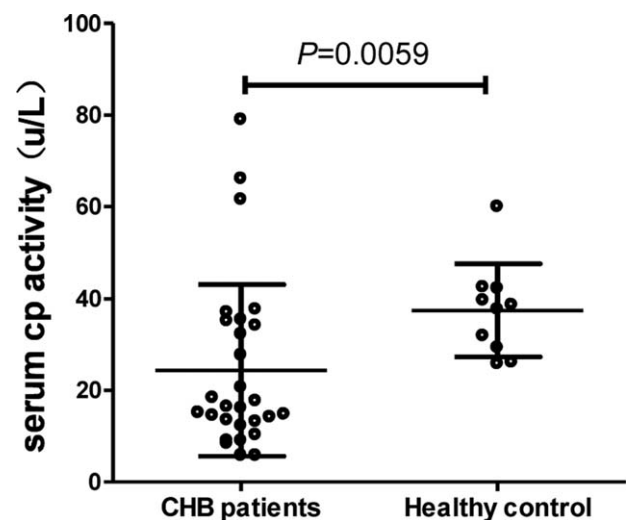


**Figure 5.** Serum ceruloplasmin (CP) activity in patients with chronic hepatitis B (CHB) and healthy controls measured by colorimetry.
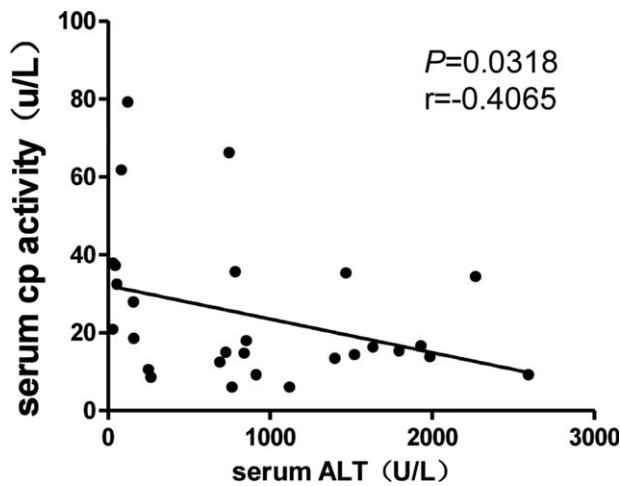
Figure 6. Spearman correlation analysis between serum ceruloplasmin (CP) activity and serum alanine transaminase (ALT) in patients with chronic hepatitis B (CHB).



Figure 8. Serum superoxide dismutase 1 (SOD1) activities in patients with chronic hepatitis B (CHB) and healthy controls measured by WST-8 assay.

It has been reported that serum Cu homeostasis is closely associated with hepatitis.[14] As such, Cu levels in a different setting of patients with chronic hepatitis have been previously reported.[15,16] However, the results are controversial. Kalkan et al reported that the Cu level was significantly higher in a viral hepatitis group, suggesting that some cytokines may have altered the level of Cu. However, Cesur et al found that the serum Cu level was not statistically different in patients with CHB compared to healthy controls. Our findings that demonstrated elevated serum TCu in patients with CHB was consistent with a previous study.[16] Persistent HBV infection may elicit a reaction from the host defense system that could have an impact on trace elements. We suggest that the elevation of serum TCu in patients with CHB is an adaptive response. Higher serum Cu levels inevitably leads to increased urinary Cu for elimination,[17] as we also found in this study, which indirectly verified the accuracy of our assay.
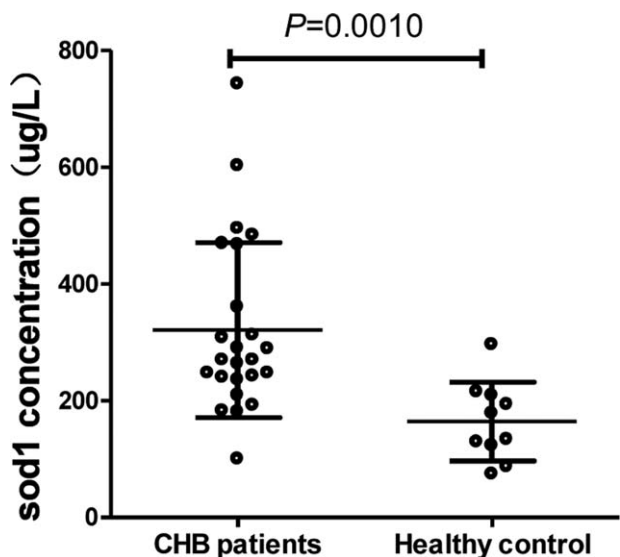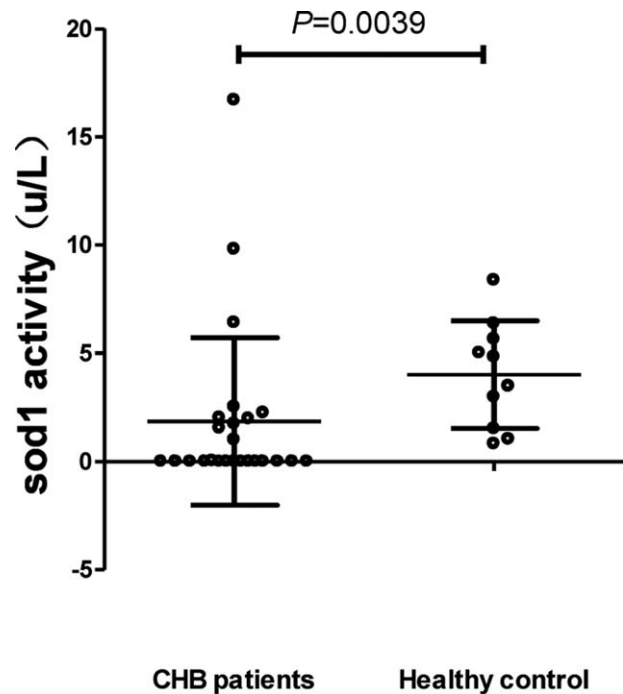
The CP is a multifunctional Cu-binding protein, and is synthesized mainly by hepatocytes then secreted into the blood as an abundant holoceruloplasmin with 6 to 7 atoms of Cu per molecule.[18] A decreased level of CP may indicate Wilson disease, Menkes disease, or aceruloplasminemia.[19] In addition, CP may function as a circulating antioxidant and an acute-phase reactant.[20] Serum CP levels have been shown to markedly increase in response to infection, inflammatory disease (eg, rheumatoid arthritis), and pregnancy.[21] In the present investigation, the concentration of CP was higher in patients with CHB compared to the control group. We reason that liver injury occurring in patients with CHB can trigger the release of CP into blood, which elevated the serum CP concentration. However, the oxidase activity of CP was significantly lower in patients with CHB than the control, and inversely correlated with serum ALT levels. Therefore, low CP activity was associated with more severe liver injury. Many studies have indicated the appearance of oxidative stress in patients with hepatitis B and hepatitis C.[22–26] It has also been reported that Cu can be dissociated from CP by ROS.[27] Such dissociation may explain the decreased CP activity in patients with CHB. Increased oxidative stress associated with HBV infection may have a negative effect on DNA damage and hepatocarcinogenesis.[28]

The SOD1 is a pivotal antioxidant enzyme in all oxygen-metabolizing tissues, and requires Cu as a cofactor. In the present study, we demonstrated a decreased activity of serum SOD1, but a higher serum concentration of SOD1, in patients with CHB. The elevation of CP and SOD1 concentration may reflect increased oxidative stress in patients with CHB, and the decreased CP and SOD1 activities may suggest a weakening of antioxidative capability in patients with CHB.

Serum $Cu^+$ is a reduced state of Cu, which is incorporated into proteins that are important for maintaining the biologic function of cells and organs. Our results have shown that serum $Cu^+$ was lower in patients with CHB than the healthy controls. We hypothesize



Figure 7. Serum superoxide dismutase 1 (SOD1) concentration in patients with chronic hepatitis B (CHB) and healthy controls measured by ELISA.

that the increased oxidative stress in patients with CHB might facilitate the conversion of $Cu^+$ to $Cu^{2+}$ through an oxidation reaction, which could subsequently result in a decreased $Cu^+$ level in patients with CHB. The serum $Cu^+$ level may indicate high oxidative stress associated with chronic HBV infection.

Moreover, the treatment of patients with CHB requires novel and more effective strategies to achieve a functional cure. The current treatments involving nucleoside/nucleotide analogues and interferons remain suboptimal. In addition to direct-acting antivirals, immunomodulators that aim to restore imbalanced immunity against HBV could be important to clear HBV infection. HBx protein, a hepatitis B viral protein associated with liver carcinogenesis, may represent a new target for HBV treatment. A previous study found that HBx-specific siRNA (siRNAx) and short hairpin RNA (shRNAx) effectively reduced the expression of HBx RNA in both HBx-transformed and HBV-producing human liver cells.[29]

In conclusion, this study showed that serum TCu was significantly higher in patients with CHB, while serum $Cu^+$ was lower. In addition, the activities of Cu-containing enzymes (CP and SOD1) in serum were lower despite the higher serum levels. Our findings suggest that HBV-infection-associated oxidative stress may reduce the antioxidative capability of CP and SOD1. Detection of serum $Cu^+$, CP, and SOD1 concentration and activity may reveal the extent of oxidative stress and relative antioxidant capabilities in patients with CHB. The results of this study may help us to understand the complexity of chronic HBV infection and physicians should be aware of the elevated oxidative stress associated with HBV infection.

## Author contributions

**Conceptualization:** Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Data curation:** Yansong Huang, Yuan Zhang, Hongqiu Cheng, Ming Han.
**Formal analysis:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng.
**Funding acquisition:** Hongqiu Cheng, Ming Han.
**Investigation:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Methodology:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Project administration:** Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Resources:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Software:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng.
**Supervision:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Validation:** Yansong Huang, Yuan Zhang, Zhexuan Lin, Hongqiu Cheng, Ming Han.
**Visualization:** Yansong Huang, Yuan Zhang, Hongqiu Cheng, Ming Han.
**Writing – original draft:** Yansong Huang, Yuan Zhang, Hongqiu Cheng.
**Writing – review & editing:** Yansong Huang, Yuan Zhang, Hongqiu Cheng.

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