Editorial Office

World Journal of Clinical Cases

Re: Revision of Manuscript (ID: No. 40837)

Dear Editor,

Thank you very much for your decision letter and the positive review of our manuscript entitled "Iron metabolism disorders in patients with hepatitis B-related liver diseases". The insightful comments and suggestions helped us to improve the manuscript.

We have read your letter and considered the comments/suggestions with care. Accordingly, we have revised the manuscript. All questions have been answered with highlighted changes in the revised manuscript. Point-to-point responses to the comment were also provided below this letter. In addition, the revised manuscript has been edited and proofread by Medjaden Bioscience Ltd.

We believe that the manuscript now is further improved. We hope that you will find that the revision is acceptable for the publication in *World Journal of Clinical Cases*.

As always, we appreciate your interest in our work, and look forward to hearing from you.

We have changed the corresponding email address to junqi_niu@163.com.

Yours sincerely,

Junqi Niu

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Point-to-Point Responses were as follows:

Reviewer #1 (Technical Comments to the Author):

The manuscript written by Gao et al. describes that serum levels of hepcidin were negatively correlated with iron deposit in the liver. Serum levels of hepcidin were also negatively correlated with HBV DNA levels and iron deposit in the liver was increased according to the progression of liver fibrosis. Since iron metabolism in HBV-related liver disease has not been well analyzed, the data are interesting and important. However, there are some concerns that need to be addressed. Major points **Comment 1：** Both age and HBV DNA levels showed statistically significant association with serum hepcidin levels. The results corrected by age should be analyzed and shown.

Response： Thank you for your suggestion. Actally we analyzed the assocation between serum hepcidin levels and HBV-DNA using multiple linear regression models, with adjustment for covariates age, total bile acid, red blood cell count, hemoglobin, hematocrit and international normalized ratio. The result of this part are showed in table.2. and in the revised manusript as below:

Method(Page 8; Line 19-20):

Correlations between variables were computed based on Spearman's correlation coefficients. Factors significanted assocated were subjected to multiple linear regression analysis.

Result (Page 11; Line 4-8):

"Subsequently, these factors were subjected to multiple linear regression analysis, which identified age (adjusted effect size=0.213, P=0.001), INR (adjusted effect size=0.198, P≤0.001) and HBV-DNA (adjusted effect size=-0.282, P<0.001) as independent factors associated with higher serum hepcidin. Notably, HBV-DNA was ranked as the most significant among all independent factors."

Comment 2. A figure showing the association between serum HBV DNA and hepcidin should be shown.

Response： Thank you for your comment. We made a figure showing the assocation between serum HBV DNA and hepcidin as below:

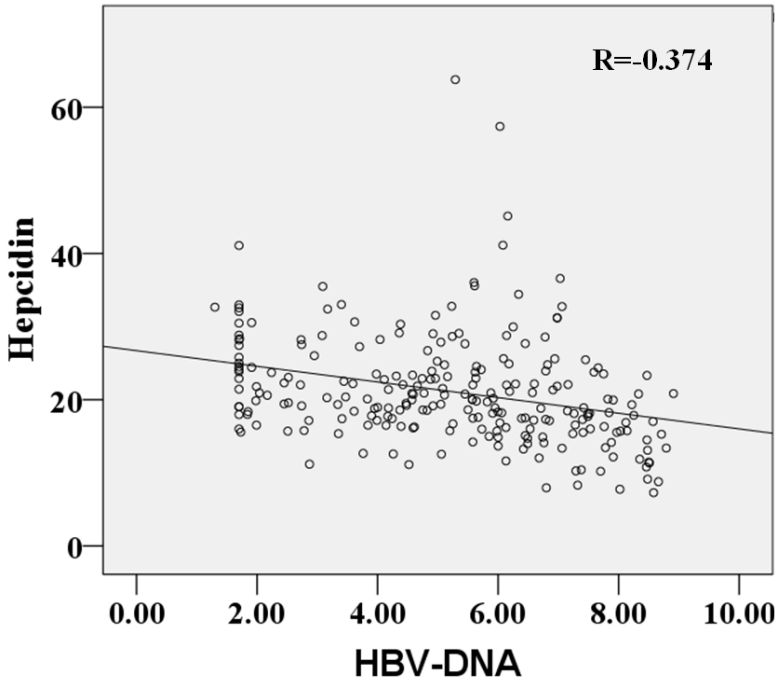

figure.5 Correlation of serum HBV DNA and hepcidin

Considering that the assocation between serum HBV DNA and hepcidin were showed in table.2. Also there were four figures which were composed of 30 graphs in the manusript. So we did add this figure in the revised manusript.

Comment 3： Minor point 1. The possible mechanism by which HBV overload or replication affect hepcidin levels should be discussed.

Response： Thank you for your suggestion. The necessary information has been added(Page 13-14) as below:

Our study further indicates that the major factors that independently associated with the altered hepcidin levels were age, HBV-DNA, and INR in patients with HBV-related diseases. The cross-sectional study design limited its role to clarify causality between HBV-DNA load and altered hepcidin levels. Wang et al. indicated that hepcidin expression was regulated by iron and inflammatory factors in HBV-infected patients, and that the virus accumulation in infected hepatocytes can affect hepcidin production[32]. However, the molecular basis by which hepcidin may alter HBV replication is

unknown. Interestingly, iron-induced hepcidin expression altered HCV replication in cultured cells [33]. Of note, other studies found no link between HBV infection and hepcidin production [34].

Comment 4. How the data lead to the conclusion that hepcidin may function as an therapeutic target for liver injury is unclear.

Response： The sentence has been deleted in the revised manuscript.

Reviewer 2

Comment 5： The title does not reflect accurately the findings from this study. Serum hepcidin levels were reported to be decreased in HBV-related disease (Fig 1) and correlated with HBV-DNA (Table 2) but no data demonstrated that hepcidin levels were correlated with liver iron deposition or in fact any serum iron parameters or markers of liver injury (Table 2). Liver iron was increased with liver fibrosis scores but hepcidin was not measured in different stages of fibrosis and was unchanged with Child-Pugh or BCLC scores (Fig 2&3). The title needs to be amended.

Response： Thank you for your suggestion. We have changed the title as per the advice, which now reads as "**Iron metabolism disorders in patients with hepatitis B-related liver diseases**".

Comment 6： The abstract results and conclusions do not reflect the data presented in manuscript. a) Serum ferritin and transferrin saturation levels increased with poorer Child-Pugh scores (Fig 2) but there was no direct evidence that they increased with fibrosis levels as stated. Child-Pugh is a classification of cirrhosis not fibrosis. Metavir score is for fibrosis. b) Hepatic iron staining levels were not positively correlated with fibrosis stage. It increased at stage 3 and 4 only but not stage 1 and 2. Amend this sentence. c) Abstract conclusions were not consistent with the results. Serum ferritin not serum iron levels were significantly higher in HBV-related disease. Serum iron was increased in CHB subjects only (Fig 1). Ferritin is an acute phase protein and may reflect the presence of hepatic inflammation or iron stores. d)

There was no direct evidence from this study that lower hepcidin levels were associated with higher liver iron staining or severity of liver injury in HBV related disease. In fact hepcidin levels were decreased in CHB compared to healthy controls and relatively increased with more severe LC or HCC disease compared to CHB. The conclusions need to be moderated.

Response：Thank you for your comments and we have revised this part in the revised manuscript as below(Page 3-4):

RESULTS: Significantly higher serum ferritin and lower serum hepcidin levels were detected in all groups of HBV-infected patients compared with healthy controls. Serum iron, total iron binding capacity and serum transferrin levels were significantly lower in patients with cirrhosis and hepatocellular carcinoma, whereas the hepcidin level was higher than that in chronic hepatitis B patients. Correlation analysis indicated that serum hepcidin was negatively correlated with HBV-DNA load ($P<0.01$). Serum ferritin and transferrin saturation levels increased proportional to the extent of liver cirrhosis and poorer Child-Pugh scores ($P<0.05$). The decreased serum iron and transferrin saturation levels were significantly correlated with a smaller hepatocellular carcinoma tumor burden according to Barcelona clinic liver cancer staging. Liver histology showed a clear increasing trend in iron deposition in the liver tissues with increased fibrosis, which became prominent at stages 3 (severe liver fibrosis) and 4 (cirrhosis).

CONLUSIONS: Iron metabolism disorders occur in patients of HBV-related liver diseases. The serum markers of iron metabolism disorders vary in different stages of HBV-related liver diseases.

Comment 7： Core tips need to be modified as outlined in comment 2 above.

Response：We have reised the core tips as below:

The relationship between hepatitis B viruses (HBV) related chronic liver diseases and levels of components in iron metabolism and the corresponding impact on liver disease severity have not been clearly described. In our study, we find that significantly higher serum ferritin and lower serum hepcidin

levels were detected in all groups of HBV-infected patients compared with healthy controls. Serum iron, total iron binding force and serum transferrin levels were significantly lower in patients with cirrhosis and hepatocellular carcinoma, whereas the hepcidin level was higher than that in chronic hepatitis B patients. In conclusion, iron metabolism disorders present in patients of HBV-related liver disease. The characteristics of iron metabolism disorders in different development stages of HBV-related liver diseases were varied.

Comment 8：In the methods section state whether all healthy controls have normal serum iron parameters and describe how liver iron levels were quantified?

Response：

Your suggestions have been well-taken, we added a sentence in the revised manuript as below(Page 7, Line 15):

"Individuals who manifested iron metabolism disorders were excluded."

Comment 9：Results 5a) p9 para 2. Fig 2 demonstrated serum ferritin and transferrin saturation changed with severity of cirrhosis not fibrosis. Also TIBC and TF levels were significantly quantified decreased with an "increased" in Child-Pugh scores. Amend text. 5b) p10 para 2. In Figure 4, it is not stated how many liver samples were tested. Were liver iron levels measured in all HBV patients? What changes were seen across the 4 HBV groups and how did the liver iron levels change with serum hepcidin? It is necessary to do this analysis before you can state that serum hepcidin changes with liver iron and injury in HBV patients.

Response：After having studied your comments, we had changed the conclusion as below (Page10, Line 6-11):

The increases in serum ferritin and transferrin saturation were proportional to the extent of liver cirrhosis and differed significantly among patients with different Child-Pugh scores (P<0.05; Fig. 2c and 2e). The TIBC and TF level were significantly decreased with a raise in Child-Pugh scores (Fig. 2f and 2d).

There were no significant differences in serum iron and hepcidin among patients with different Child-Pugh scores (P>0.05; Fig. 2a and 2b).

(Page10--11):

A total of 29 patients were selected for iron staining, including 5 CHB patients (4 males) at S0, 8 (5 males) at S1, 4 (3 males) at S2, 7 (6 males) at S3, and 5 (2 males) at S4. There were no significant differences in the median (P25-P75) ages among the different stage groups (P=0.122).

We also change the figure 4 legend as below(Page 27-28):

Figure 4. Iron deposition in liver tissues with fibrosis of different stages.

Perls' staining of iron appears as red granular particles in the liver cells (×400 magnification.) A-E show different stages of liver fibrosis, respectively. Liver fibrosis was staged using the METAVIR scoring system, which consists of five stages: S0 (no fibrosis, n=5), S1 (portal fibrosis without septa, n=8), S2 (portal fibrosis with rare septa, n=4), S3 (portal fibrosis with many septa, n=7), and S4 (cirrhosis, n=5). Markedly increased iron deposition was observed in the severe liver fibrosis (S3) and cirrhosis (S4) groups, but not in groups S0-S2. F shows significantly higher average iron retention with severe fibrosis (S3: 23.7%) and cirrhosis (S4:43.6%) compared to that with no or mild fibrosis (S0: 5.2%, S1: 7.9%; S2: 8.5%). Statistically significant differences in iron staining were observed among patients with severe fibrosis and cirrhosis (P<0.05).

The sentence that "serum hepcidin changes with liver iron and injury in HBV patients" has been deleted in the revised manuscript.

Comment 10： Discussion 6a) p12 para 2. Again there is no direct evidence to show "that a decreased serum hepcidin levels correlated with excessive iron accumulation in patients with HBV-related liver disease."

Response： Your suggestions have been well-taken, the sentences had been deleted in the revised manuscript and the discussion was modified as below(Page13-14):

"In addition to ferritin and transferrin, hepatocytes also produce and secret hepcidin, an acute phase reactant protein that may negatively regulate the

endogenous iron level and reduce the release of iron from cells by interacting with the cellular iron exporter ferroportin that leads to subsequent internalization and degradation [30, 31]. Our study further indicates that the major factors that independently associated with the altered hepcidin levels were age, HBV-DNA, and INR in patients with HBV-related diseases. The cross-sectional study design limited its role to clarify causality between HBV-DNA load and altered hepcidin levels. Wang et al. indicated that hepcidin expression was regulated by iron and inflammatory factors in HBV-infected patients, and that the virus accumulation in infected hepatocytes can affect hepcidin production[32]. However, the molecular basis by which hepcidin may alter HBV replication is unknown. Interestingly, iron-induced hepcidin expression altered HCV replication in cultured cells [33]. Of note, other studies found no link between HBV infection and hepcidin production [34]."

Comment 11： 6b) p13 para 3. In the conclusion the text needs to be changed as outlined before (2c and 2d).

Response： After having studied your comments, we had changed the conclusion as below(Page 14, Line 10-12):

"In conclusion, iron metabolism disorders can occur in patients with HBV-related liver disease. The serum markers of iron metabolism disorders vary in different stages of HBV-related liver diseases."

Comment 12： Other comments 7a) Change total iron binding force to total iron binding capacity (Abstract para 3; p7, para 3; p9 para 1).

Response： Thank you for your attention to details, this has been revised in the revise manuscript.

Comment 13: 7b) Fig 1-3. Include units on y-axis. State whether results are expressed as mean ± SEM or median ± range; n=? 7c) Define what fibrosis stage (S0-S4) is shown in Figs 4a-e.

Response：

As suggested, units on y-axis had been added a added in the revised figrures.

The results are expressed as mean ± SEM in all figures and we had state that in the revised manuscript as below(Page 24-26):

"Figure 1. The mean level of serum iron markers and the standard error mean(SEM) of them among four groups.

Figure 2. Serum iron markers among liver cirrhosis patients with different Child-Pugh classes (mean± SEM).

Figure 3. Serum iron markers (mean±SEM) among HCC patients with different Barcelona Clinic Liver Cancer (BCLC) stages. "

We had added the Liver fibrosis stage definition in the revised manuscript as below(Page 27):

"Figure 4. Iron deposition in liver tissues with fibrosis of different stages. Perls' staining of iron appears as red granular particles in the liver cells (×400 magnification.) A-E show different stages of liver fibrosis, respectively. Liver fibrosis was staged using the METAVIR scoring system, which consists of five stages: S0 (no fibrosis, n=5), S1 (portal fibrosis without septa, n=8), S2 (portal fibrosis with rare septa, n=4), S3 (portal fibrosis with many septa, n=7), and S4 (cirrhosis, n=5). Markedly increased iron deposition was observed in the severe liver fibrosis (S3) and cirrhosis (S4) groups, but not in groups S0-S2. F shows significantly higher average iron retention (mean±SEM) with severe fibrosis (S3: 23.7%) and cirrhosis (S4:43.6%) compared to that with no or mild fibrosis (S0: 5.2%, S1: 7.9%; S2: 8.5%). Statistically significant differences in iron staining were observed among patients with severe fibrosis and cirrhosis (P<0.05).

Comment 14: In Fig 4f state whether the results are expressed as mean ± SEM or median ± range; n=?

Response: Thank you for your suggestion. The results are expressed as mean

± SEM in all figure 4 and we had state that in the revised manuscript as below:

"Figure 4. Iron deposition in liver tissues with fibrosis of different stages.

Perls' staining of iron appears as red granular particles in the liver cells (×400 magnification.) A-E show different stages of liver fibrosis, respectively. Liver fibrosis was staged using the METAVIR scoring system, which consists of five stages: S0 (no fibrosis, n=5), S1 (portal fibrosis without septa, n=8), S2 (portal fibrosis with rare septa, n=4), S3 (portal fibrosis with many septa, n=7), and S4 (cirrhosis, n=5). Markedly increased iron deposition was observed in the severe liver fibrosis (S3) and cirrhosis (S4) groups, but not in groups S0-S2. F shows significantly higher average iron retention (mean±SEM) with severe fibrosis (S3: 23.7%) and cirrhosis (S4:43.6%) compared to that with no or mild fibrosis (S0: 5.2%, S1: 7.9%; S2: 8.5%). Statistically significant differences in iron staining were observed among patients with severe fibrosis and cirrhosis ($P<0.05$)."