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Hepcidin Exacerbates Iron Metabolism Imbalance in Septic Mice

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Purpose: Sepsis is a life-threatening condition associated with acute organ dysfunction. Iron is an essential trace element for multicellular organisms and almost all microorganisms, and its role in sepsis has been increasingly recognized. The aim of this study was to investigate the changes in iron metabolism in caecal ligation and puncture solution (CLP) -induced septic mice and the effects of hepcidin pretreatment on serum inflammatory marker levels and liver iron metabolism in CLP-induced septic mice.

Methods: C57BL/6 mice were given normal saline, CLP (peritonitis model) or 100 μg of hepcidin via intraperitoneal injection. The experimental animals were divided into 4 groups: the control group, model group (CLP), hepcidin pretreatment Groups CLP+hepcidin-2h and CLP+hepcidin-24 h. Blood samples were collected at 6, 12 and 24 hours after CLP surgery, and the mice were euthanized and livers were obtained.

Results: ELISA revealed that hepcidin pretreatment, especially 2 hours in advance ($p<0.01$), increased the serum hepcidin, TNF-a and IL-6 in CLP-induced septic mice; the serum iron content of CLP-related septic mice decreased ($P<0.01$), while the liver iron content increased ($P<0.01$); Hepcidin pretreatment reduced the serum iron ($P<0.05$) at 6 h and 12 h and liver iron concentrations ($P<0.01$) at 6 h, 12 h and 24 h in CLP-related septic mice. Western blotting revealed that the hepatic iron absorption-related proteins transferrin receptor-2 (TFR2), ZRT/IRT-like protein 14 (ZIP14) and divalent meta lion transporter-1 (DMT1) were elevated ($P<0.01$); The iron-exporting protein ferroportin (SLC40A1) was decreased ($P<0.01$) throughout CLP and CLP+hepcidin sepsis. Compared with CLP group, the protein expressions in the CLP+ hepcidin-2 h group were more obvious than that in the CLP+ hepcidin-24 h group.

Conclusion: Hepcidin has proinflammatory effect. Hepcidin exacerbates iron metabolism imbalances in sepsis by influencing the expression of iron absorption-related proteins and iron export-related proteins.

Keywords: hepcidin, sepsis, iron metabolism, SLC40A1, liver

Introduction

Sepsis is a life-threatening disease that results in a dysregulated host response to an infection and is associated with acute organ dysfunction and a high risk of death.[1] In recent years, the role of trace element metabolism dysregulation in the pathogenesis of sepsis has been increasingly recognized. Iron is an essential trace element for multicellular organisms and nearly all microorganisms.[2]

In the human body, iron-binding proteins are components of proteins or enzymes involved in vital biological processes and are indispensable to all cells. In addition, iron is also a critical biological element of life in microbes and has been shown to increase the virulence of bacteria or accelerate the growth of pathogens.[3] As a defensive measure, the host needs to limit iron availability to pathogens by increasing iron transporters and iron absorption and decreasing iron output. This results in a restriction of free iron in circulation, which increases intracellular iron.[4] Excess iron in the cytoplasm may aggravate inflammation, trigger cell death, and even lead to multiple-organ damage and death. Reduced iron availability caused by iron retention is an important contributor to anaemia and is associated with a poorer outcome.[5]

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The liver is an important line of defence against microbes and plays a crucial role in sepsis.[6] Hepcidin is an acute-phase peptide hormone secreted by the liver in response to iron loading and inflammation.[7] Hepcidin binds to its receptor, the iron exporter ferroportin, hepcidin and its receptor ferroportin are internalized together and trafficked to lysosomes where both are degraded, thus blocking iron efflux from cells into plasma.[8] Therefore, hepcidin is now considered to be the most important factor controlling iron absorption.

As the sole exporter of intracellular iron, ferroportin is a prominent regulator of the plasma iron concentration. Posttranscriptional regulation of ferroportin is governed by hepcidin.[9] During sepsis, the expression of ferroportin is downregulated.[10]

Therefore, the hepcidin-ferroportin axis plays an important role in the imbalance of iron metabolism in sepsis. The aim of this study was to investigate the changes in iron metabolism in CLP-induced septic rats and the effects of hepcidin pretreatment on the serum inflammatory marker levels and liver iron metabolism in CLP-induced septic rats.

Materials and Methods

Mice and Induction of Sepsis

All animal experiments performed for this study were approved by the Experimental Animal Ethics Committee of Kangtai Medical Laboratory Service Hebei Co., LTD (IACUC approval number MDL2022-12-1). All procedures related to animals were conducted according to the Regulations on the Administration of Laboratory Animals of the State Science and Technology Commission of China. Male, 8 to 10-week-old C57BL/6 mice (a total of 36 mice) were procured from Spf (Sipeifu, Beijing, China) Biotechnology Company and were used throughout this study. Polymicrobial sepsis was induced by caecal ligation and puncture (CLP).[11,12] After adaptive feeding (approximately 30 g), the mice were fasted for 12 hours. After anaesthesia, the mice were fixed in the supine position and given abdominal disinfection and hair removal. CLP features ligation below the ileocecal valve after midline laparotomy, followed by needle puncture of the caecum and extrusion of a small amount of faecal matter. Finally, the peritoneum and skin were sutured. We injected C57BL/6 mice intraperitoneally with hepcidin (100 μg) or PBS at different time points before CLP administration.[13] Human hepcidin-25 (B10902, Bachem, Switzerland).

Experimental Group

The experimental animals were divided into 4 groups with 9 mice in each group: the control group (PBS), model group (CLP), hepcidin pretreatment Group 2 hours before CLP (CLP+hepcidin-2 h), and hepcidin pretreatment Group 24 hours before CLP (CLP+hepcidin-24 h). Mice in the control group and model group were injected with PBS; mice in the 2-hour or 24-hour pretreatment group were injected with 100 μg of hepcidin 2 h or 24 h prior to CLP induction. The survival of the mice in each group within 24 h was recorded. Blood samples were collected at 6, 12 and 24 hours after modelling, and serum was separated from 3 mice in each group. The liver tissues of mice in the respective experimental groups (control group, CLP, CLP+hepcidin-2 h or CLP+hepcidin-24 h) were stored at −80 °C.

Assessment of Cytokine Levels

Serum hepcidin, IL-6, TNF-α, iron and liver iron levels were measured using commercial kits according to the manufacturer's instructions. Enzyme-linked immunosorbent assay (ELISA) kits for mouse Hepcidin (MM-44770M1, MEIMIAN, China), IL-6 (MM-0163M1, MEIMIAN, China) and TNF-α (MM-0132M1, MEIMIAN, China). Iron Colorimetric Assay Kit (E1042, Applygen, China).

Western Blotting

The expression of proteins TFR2 (anti-TFR2 antibody, ab80194, Abcam, UK), ZIP14 (anti-ZIP14 antibody, ab106568, Abcam, UK), DMT1 (anti-DMT1 antibody, ab55735, Abcam, UK) and SLC40A1 (anti-SLC40A1 antibody, PA5-22993, Invitrogen, America) were detected by Western blotting. 36 mice were euthanized and livers were obtained. Liver tissue (50 mg) was solubilized in RIPA buffer (Cell Signaling Technology) on ice for 20 min. The protein concentration was subsequently determined using a BCA protein assay (#7780, Cell Signaling Technology, America), as recommended by

the manufacturer. Equal quantities of protein were separated via 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with 5% (wt/vol) nonfat dry milk in TBST solution for 1 h at room temperature, the membranes were incubated first with the primary antibody overnight at 4 °C and then with the HRP-conjugated secondary antibody (1:10000) for 70 minutes at 37 °C. The membranes were probed by using specific antibodies and visualized with an ECL detection system. The strips were analysed, and the OD values were calculated.

Statistical Analysis

The statistical software SPSS V.26.0 and GraphPad Prism V.9.5.0 were used for statistical analysis. The Kolmogorov–Smirnov test was used to test whether continuous numerical variables obeyed a normal distribution. Normally distributed data are expressed as the mean ± standard deviation; if continuous numerical variables were not normally distributed, the data are expressed as the median and upper and lower quartiles [M (P25, P75)]. Comparisons were performed with the rank sum test. One-way ANOVA was used for continuous normality data, multiple LSD comparisons were used for the same time points in different groups, and one-way ANOVA was used for different time points in the same group. $P<0.05$ was considered to indicate statistical significance.

Results

Hepcidin Pretreatment Increases the Serum Hepcidin Concentration in CLP-Related Septic Mice

Compared with the control group, serum hepcidin in CLP was significantly increased at 6 h, 12 h ($P<0.01$), serum hepcidin in CLP+hepcidin-2 h and CLP+hepcidin-24 h groups were significantly increased at 6 h, 12 h and 24 h ($P<0.01$). The serum hepcidin concentration in the CLP+hepcidin-2 h group was significantly greater than that in the CLP group at 6 h, 12 h and 24 h ($P<0.01$). The serum hepcidin concentration in the CLP+hepcidin-24 h group was greater than that in the CLP group at 6 h, 12 h and 24 h ($P <0.05$). These data suggested that hepcidin pretreatment increases the serum hepcidin concentration in septic mice. (Figure 1A; Supplementary Table S1).

Hepcidin Pretreatment Increases the Serum TNF-α and IL-6 Concentrations in CLP-Induced Septic Mice

Compared with those in the control group, the serum IL-6 and TNF-α levels in the CLP, CLP+hepcidin-2 h and CLP +hepcidin-24 h groups were significantly greater at 6 h, 12 h and 24 h ($P<0.01$). The serum IL-6 and TNF-α levels in the CLP+hepcidin-2 h group were significantly greater than those in the CLP group at 6 h, 12 h and 24 h ($P<0.01$). The serum IL-6 and TNF-α levels in the CLP+hepcidin-24 h group were greater than those in the CLP group at 6 h and 12 h ($P <0.01$), but there was no significant difference in the serum IL-6 and TNF-α levels between the two groups at 24 h ($P>0.05$). These data suggested that hepcidin increases the serum inflammatory marker levels in septic mice (Figure 1B and C; Supplementary Table S1).

Hepcidin Pretreatment Reduces the Serum Iron Concentration in CLP-Related Septic Mice

Compared with those in the control group, the serum iron concentrations in the CLP, CLP+hepcidin-2 h and CLP +hepcidin-24 h groups were significantly lower at 6 h, 12 h and 24 h ($P<0.01$). Serum iron in CLP+hepcidin-2 h group was decreased at 6 h and 12 h compared with CLP group ($P<0.05$), and there was no significant difference in serum iron reduction at 24 h compared with CLP group ($P>0.05$). There was no significant difference in the serum iron concentration between the CLP+hepcidin-24 h group and the CLP group ($P>0.05$). The serum iron concentration in the CLP +hepcidin-2 h group at 6 h was significantly lower than that in the CLP+hepcidin-24 h group ($p<0.01$), and there was no significant difference between the two groups at 12 h or 24 h (Figure 1D; Supplementary Table S1).

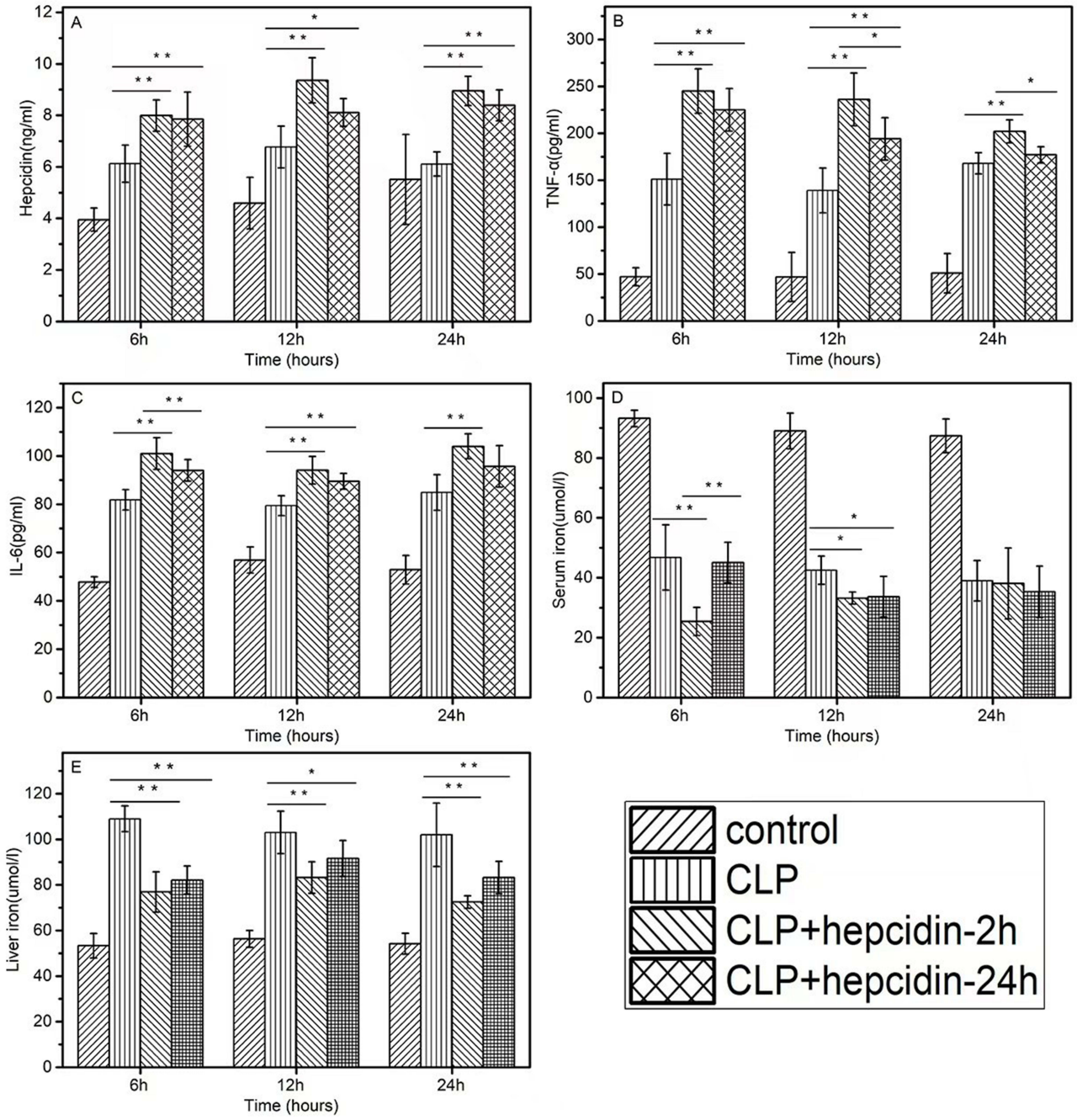

Figure 1 Serum hepcidin, TNF-α, IL-6, iron and liver iron levels were determined via ELISA in CLP-induced septic mice. The 36 experimental animals were divided into 4 groups with 9 in each group: the control group, model group (CLP), hepcidin pretreatment Group 2 hours before CLP (CLP+hepcidin-2 h), and hepcidin pretreatment Group 24 hours before CLP (CLP+hepcidin-24 h). Blood samples were collected at 6, 12 and 24 hours after modelling. (**A**) Serum hepcidin (6 h n=3, 12 h n=3, 24 h n=3); (**B**) TNF-α (6 h n=3, 12 h n=3, 24 h n=3); (**C**) IL-6 (6 h n=3, 12 h n=3, 24 h n=3); (**D**) Serum iron (6 h n=3, 12 h n=3, 24 h n=3); (**E**) Liver iron (6 h n=3, 12 h n=3, 24 h n=3). There were statistical differences between the control group and the model group or the hepcidin pretreatment group in each figure ($p<0.01$), except that there was no statistical difference between the control group and the model group at 24h in Figure A ($p>0.05$). The significant difference between the model group and hepcidin pretreatment groups at the same time point (6, 12 and 24 hours) is marked with * above the bar chart. *$p<0.05$, **$p<0.01$.

Hepcidin Pretreatment Reduces Liver Iron Levels in CLP-Related Septic Mice

Compared with those in the control group, the liver iron concentrations in the CLP, CLP+hepcidin-2 h and CLP +hepcidin-24 h groups were significantly greater at 6 h, 12 h and 24 h ($P<0.01$). The liver iron concentrations in the CLP+hepcidin-2 h group ($P<0.01$) and CLP+hepcidin-24 h group ($P<0.05$) were lower than those in the CLP group at

6 h, 12 h and 24 h. The liver iron concentration in the CLP+hepcidin-2h group was lower than that in the CLP+hepcidin-24h group, but the difference was not statistically significant (P>0.05) (Figure 1E; Supplementary Table S1).

Effects of Hepcidin on Liver Iron Absorption and Iron Transport in CLP-Related Septic Mice. TFR2 Expression in the Liver Was Elevated Throughout CLP-Related Sepsis and CLP+ Hepcidin Sepsis

Compared with that in the control group, TFR2 expression in the CLP, CLP+hepcidin-2 h and CLP+hepcidin-24 h groups was significantly increased at 6 h, 12 h and 24 h (P<0.01). The expression of TFR2 in the CLP+hepcidin-2 h group was greater than that in the CLP group at the above 3 time points (P<0.01). The expression of TFR2 in the CLP+hepcidin-24 h group was greater than that in the CLP group at 6 h (P<0.01), and there was no significant difference between the CLP and CLP+hepcidin-24 h groups at 12 h and 24 h (P>0.05). Thus, the data showed that the expression of TFR2 increased more significantly in the CLP+ hepcidin-2 h group than in the CLP+ hepcidin-24 h group in septic mice pretreated with hepcidin (Figure 2A, I–III; Supplementary Table S2).

Zip14 Expression in the Liver Was Elevated Throughout CLP-Related Sepsis and CLP +hepcidin Sepsis

Compared with that in the control group, Zip14 expression in the CLP, CLP+hepcidin-2 h and CLP+hepcidin-24 h groups was significantly increased at 6 h, 12 h and 24 h (P<0.01). The expression of Zip14 in the CLP+hepcidin-2 h group was greater than that in the CLP group at the above 3 time points (P<0.01). The expression of Zip14 in the CLP+hepcidin-

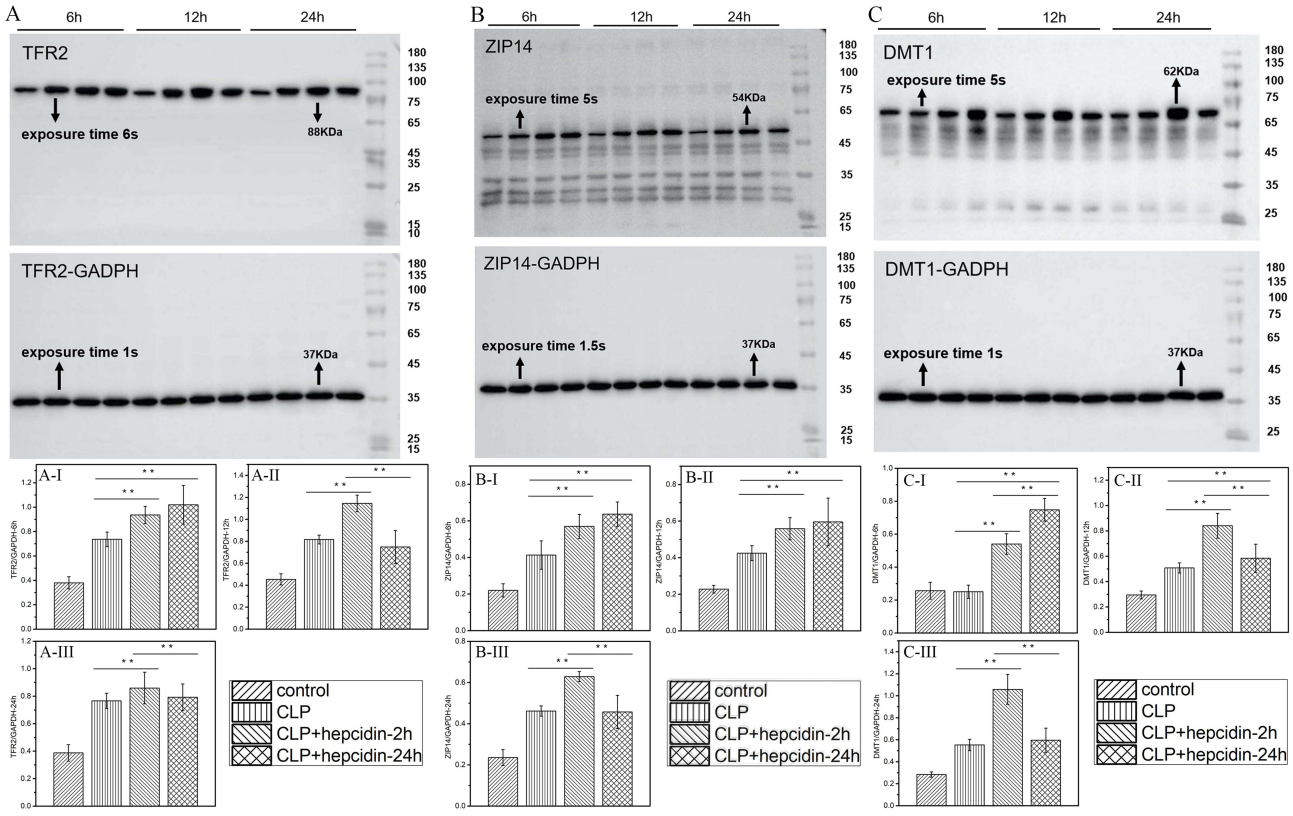

Figure 2 Effects of hepcidin on liver iron absorption protein in CLP-related septic mice. Liver tissue was solubilized. The expression of proteins (TFR2, ZIP14, DMT1) was detected by Western blotting. The strips were analysed, and the OD values were calculated. The significant difference between the model group and hepcidin pretreatment groups at the same time point (6, 12 and 24 hours) is marked with ** above the bar chart. (**A**) I–III) TFR2 (6 h n=3, 12 h n=3, 24 h n=3); (**B**) I–III) ZIP14 (6 h n=3, 12 h n=3, 24 h n=3); (**C**) I–III) DMT1 (6 h n=3, 12 h n=3, 24 h n=3). ** p<0.01.

24 h group was greater than that in the CLP group at 6 h and 12 h (P<0.01), and there was no significant difference between the CLP and CLP+hepcidin-24 h groups at 24 h (P>0.05) (Figure 2B, I–III; Supplementary Table S2).

DMT1 Expression in the Liver Was Elevated Throughout CLP-Related Sepsis and CLP +hepcidin Sepsis

The expression of DMT1 in the CLP group was greater than that in the control group at 12 h and 24 h (P<0.01). Compared with that in the control group, the expression of DMT1 in the CLP+hepcidin-2 h and CLP+hepcidin-24 h groups was significantly greater at 6 h, 12 h and 24 h (P<0.01). Within 24 h, the expression of DMT1 in the CLP+hepcidin-2 h group gradually increased, and the expression level was greater than that in the CLP group (P<0.01). The expression of DMT1 in the CLP+hepcidin-24 h group was greater than that in the CLP group at 6 h (P<0.01), but the expression of DMT1 at 12 h and 24 h was not significantly greater than that in the CLP group. Therefore, the data showed that the expression of DMT1 in septic mice pretreated with hepcidin 2 hours before modelling was greater than that in mice pretreated with hepcidin 24 hours before modelling (Figure 2C, I–III; Supplementary Table S2).

SLC40A1 Expression in the Liver Was Decreased Throughout CLP-Related Sepsis and CLP+ Hepcidin Sepsis

Compared with that in the control group, SLC40A1 expression in the CLP, CLP+hepcidin-2 h and CLP+hepcidin-24 h groups was significantly lower at 6 h, 12 h and 24 h (P<0.01). SLC40A1 expression in the CLP+hepcidin-2 h group at the above 3 time points was significantly lower than that in the CLP group (P<0.01). The expression of SLC40A1 in the CLP+hepcidin-24 h group at 6 h and 24 h was significantly lower than that in the CLP group (P<0.01). The expression of SLC40A1 in the CLP+hepcidin-24 h group at 12 h was not significantly lower than that in the CLP group (Figure 3A, I–III).

Discussion

Hepcidin pretreatment increased the serum hepcidin, TNF-a and IL-6 concentrations in CLP-induced septic mice. The present study demonstrated that the serum iron content of CLP-related septic mice decreased, while the liver iron content increased. Hepcidin-pretreated CLP-induced septic mice exhibited a decrease in the serum and liver iron content, but the level was still significantly greater than that in normal mice. The iron absorption-related proteins TFR2, ZIP14 and DMT1 were increased in the liver tissue of CLP-sepsis and CLP+hepcidin septic mice. The iron-exporting protein ferroportin (SLC40A1) in the liver is decreased throughout CLP-related sepsis and CLP+hepcidin sepsis. Changes in the expression of these proteins were more obvious in CLP-related septic mice pretreated with hepcidin.

Hepcidin is a systemically acting iron-regulatory peptide hormone and the only known natural ferroportin (SLC40A1) ligand. Ferroportin is regulated by the hormone hepcidin. Ferroportin is the only known cellular iron exporter in vertebrates and is the conduit through which iron is delivered into plasma.[14] Iron homeostasis is maintained by the hepcidin-ferroportin axis, which controls the intestinal absorption of iron, as well as internal iron recycling and systemic distribution.[14] As a host defence mediator, hepcidin increases in response to infection and inflammation, blocking iron delivery through ferroportin to blood plasma and thus limiting iron availability to invading microbes.[14,15]

Several studies have shown that iron metabolism changes during sepsis, iron uptake into cells increases, and iron output decreases.[2] The serum ferritin Fe^{3+} concentration is an independent risk factor for death in sepsis patients, and the $Fe3^{+}$ concentration is significantly decreased in the deceased sepsis.[16] In the present study, the serum iron content of septic mice decreased, and the liver iron content increased, which was consistent with the findings of Stefanova et al.[17] We found that the serum iron and liver iron levels in hepcidin-pretreated CLP-induced septic mice were lower than those in CLP-induced septic mice. The effects of hepcidin on systemic inflammation and local inflammation and whether different organs have different effects and mechanisms need to be further studied. In patients with sepsis, elevated hepcidin levels inhibit iron export, resulting in decreased serum iron levels. However, iron retention causes intracellular iron overload, which is associated with tissue damage and multiple organ dysfunction syndrome (MODS).[5]

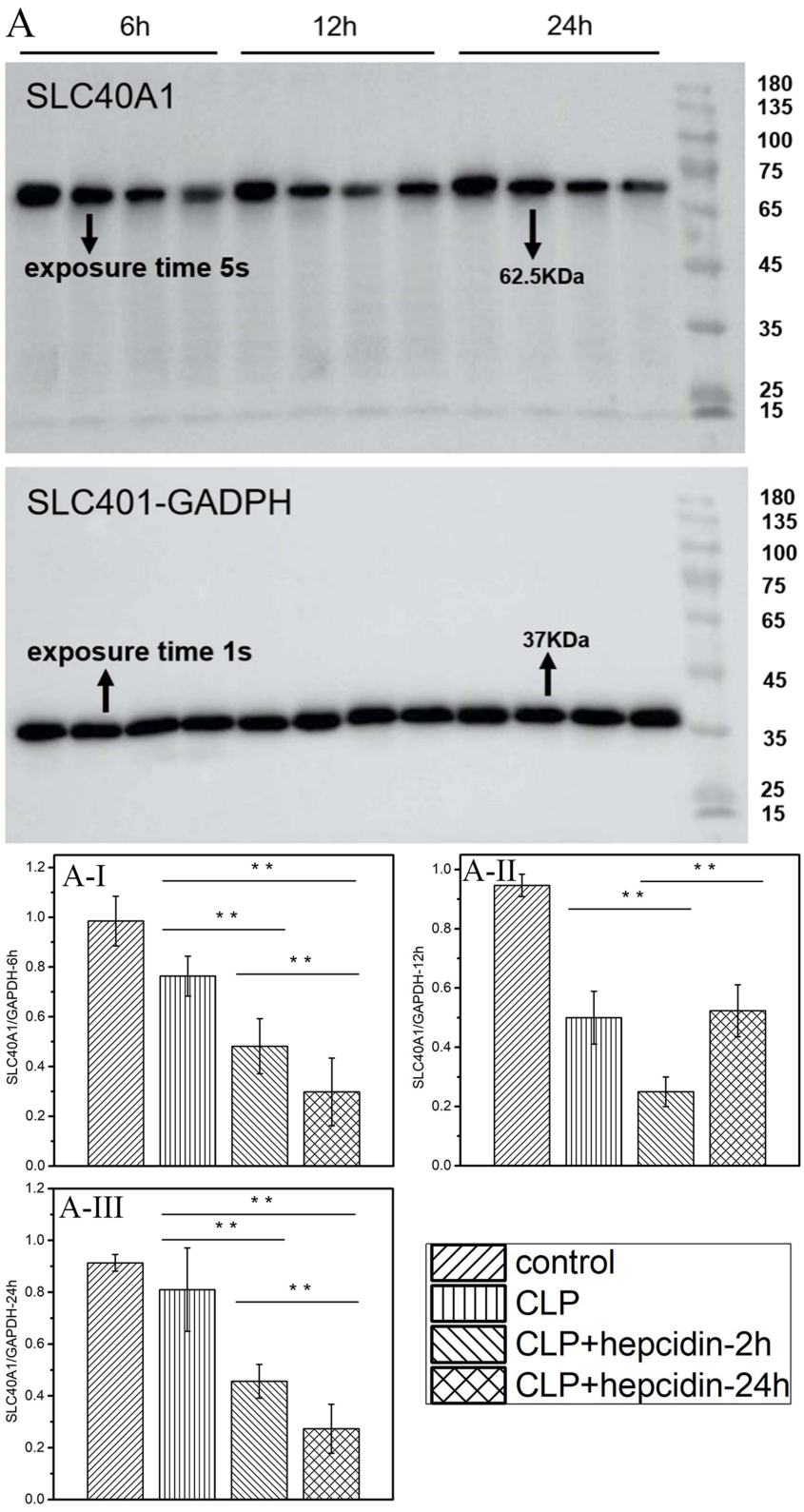

Figure 3 Effects of hepcidin on liver iron transporters in CLP-related septic mice. Liver tissue was solubilized. The expression of proteins (SLC40A1) was detected by Western blotting. The strips were analysed, and the OD values were calculated. The significant difference between the model group and hepcidin pretreatment groups at the same time point (6, 12 and 24 hours) is marked with ** above the bar chart. (**A, AI–III**) SLC40A1 (6 h n=3, 12 h n=3, 24 h n=3) **p<0.01.

Transferrin receptor (TFR) mainly mediates transferrin-bound iron uptake, whereas DMT1 and ZIP14 mediate non-transferrin-bound iron (NTBI) uptake at the apical side of hepatocytes.[18,19] Hepatic iron uptake depends on transferrin receptor 1/2 (TFR1 and TFR2), DMT1 and ZIP14.[20–22] Iron efflux is regulated by the iron-exporting protein ferroportin (SLC40A1).[14] TfR1 is expressed almost ubiquitously, but its level is particularly low in the liver.[23] In contrast, TfR2 is expressed almost exclusively in the liver. During the same period, the expression of TfR1 in the liver decreases during development, while the expression of TfR2 increases dramatically.[24] In the present study, we detected elevated TFR2 expression in the livers of CLP-related septic mice and CLP+ hepcidin-induced septic mice. At three time points in the study, the expression of TFR2 in the CLP+hepcidin-2 h group was greater than that in the CLP sepsis group. Compared with that in the CLP+ hepcidin-24 h group, the expression of TFR2 in the CLP+ hepcidin-2 h group was significantly greater in hepcidin-pretreated mice with sepsis.

Among human tissues, ZIP14 is ubiquitously expressed, with the most abundant expression in the liver, pancreas, and heart.[25] In sepsis, the reduction in ZIP14 expression in the gut is the opposite of the increase in ZIP14 generation in the liver.[26] The regulatory mechanism of ZIP14 differs among various tissues and organs. Wessels et al showed that ZIP14 expression in the liver was strongly elevated in mice with CLP-induced sepsis, and ZIP14 mRNA was increased by 9 h after CLP.[27] DMT1 protein levels are downregulated in the livers of iron-loaded rats and upregulated in the livers and hearts of iron-deficient rats.[28] In this study, we found that ZIP14 and DMT1 expression was elevated in the livers of mice with CLP-related sepsis and CLP+ hepcidin-related sepsis. At the three time points in the present study, the expression of ZIP14 in the septic mice pretreated with hepcidin 2 hours in advance was greater than that in the CLP septic mice. With the change in time within 24 hours, compared with septic mice pretreated with hepcidin 24 hours earlier, septic mice pretreated with hepcidin 2 hours earlier showed more elevated expression of ZIP14 and DMT1.

Jin Fang et al reported that the expression of ferroportin was significantly reduced in a rat model of LPS-induced endotoxemia.[10] We obtained similar results for the changes in SLC40A1 in CLP-induced septic mice. The expression of SLC40A1 in the liver of the CLP+hepcidin-2 h group decreased within 24 h and was lower than that in the CLP-induced septic mice at different time points. These data indicated that hepcidin influenced the expression of iron absorption-related proteins and iron export-related proteins.

A present prospective clinical study showed that increased hepcidin levels were associated with the diagnosis of septic shock.[29] Hepcidin levels at admission were correlated with 180-day mortality in the sepsis group, and hepcidin levels were significantly higher in the septic than in the non-septic patients.[29] An article exploring the role of hepcidin in the diagnosis of sepsis and septic shock in children showed that hepcidin had a sensitivity of 95.6% and a specificity of 100% in differentiating the intensive care control group from the sepsis group.[30] The sensitivity of WBC, CRP and PCT was lower than that of hepcidin, while the sensitivity of IL-6 was higher than that of hepcidin. The specificity of PCT and IL-6 was the same as that of hepcidin, while that of WBC and CRP was lower than that of hepcidin.[30] The present study confirmed that Hepcidin had proinflammatory effect and exacerbated iron metabolism imbalance in mice with sepsis. For sepsis, hepcidin may be a valuable diagnostic tool, and the hepcidin-ferroportin axis may be meaningful intervention target.

This study has several limitations. First, the expressions of cytokines and proteins were mainly observed at 6, 12, and 24 hours after modelling. If the observation time is extended, we do not know what the experimental results will be over time. Second, we did not analyse the histopathological characteristics.

Conclusions

In conclusion, this study suggested that hepcidin has a proinflammatory effect and exacerbates iron metabolism imbalance in sepsis by influencing the expression of iron absorption-related proteins and iron export-related proteins. Iron metabolism indicators have potential application prospects in the prognostic prediction and treatment evaluation of sepsis and are expected to provide actionable targets for therapeutic intervention.

Abbreviations

CLP, cecal ligation and puncture; SLC40A1, ferroportin; TFR, transferrin receptor; Zip14, ZRT/IRT-like protein 14; DMT1, divalent metal ion transporter-1; TNF-a, tumor necrosis factor-alpha; IL-6, Interleukin-6; NTBI, non-transferrin-

bound iron; PBS, phosphate buffer saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OD, Optical Density; MODS, multiple organ dysfunction syndrome.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

All animal experiments were performed following the protocol approved by the Animal Ethical committee (IACUC approval number MDL2022-12-1).

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Disclosure

The authors declare that they have no competing interests.

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