Original Article
The effect of hepcidin in rats with renal ischemia/reperfusion injury

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Abstract: Objective: This study sought to investigate the effectiveness of hepcidin in renal ischemia/reperfusion injury by using a rat model of renal IRI. Methods: In our study, male Sprague-Dawley rats were divided into a hepcidin-treated group and a control group before establishing the animal models. According to the difference of the modelling methods (renal pedicle occlusion for 45 minutes or not) and renal reperfusion time, the rats were then respectively divided into four subgroups: sham, IRI 4 h, IRI 12 h, and IRI 24 h. After the establishment of the IRI model, the rats were killed to determine renal function, histology, iron metabolism indexes in plasma and tissues, and the expression level of hepcidin and ferroportin-1. Results: The results indicated that the levels of serum creatinine, blood urea nitrogen and serum iron, the renal iron content, and the kidney injury score were significantly decreased in the hepcidin group (P<0.05). The serum hepcidin and the splenic iron content were significantly increased while the duodenal iron content was significantly decreased in the hepcidin group (P<0.05). Hepcidin expression in the liver and ferroportin-1 expression in the kidneys were significantly decreased in the hepcidin group (P<0.05). Conclusion: Hepcidin has a reno-protective effect in renal IRI by possibly promoting iron intake in the spleen, inhibiting iron absorption and exportation in the duodenum, alleviating the degree of serum iron, and reducing renal iron accumulation in the renal IRI.

Keywords: Ischemia/reperfusion injury, hepcidin, ferroportin, iron metabolism disorder, rat model

Introduction
Previous studies have demonstrated that iron is one of the most important factors in apoptosis during renal ischemia/reperfusion injury (IRI) [1, 2]. These studies and the present study have also indicated that an iron metabolism disorder within the kidneys during renal IRI exists [2]. Renal IRI accompanied the increase in renal iron content, possibly because an iron metabolism disorder and iron deposition take place in the kidneys during renal IRI, further aggravating the kidney injury by direct and indirect toxic reactions involving iron [3]. However, the exact mechanism leading to iron metabolism disorder during renal IRI is unclear. Previous studies reported that tissue ischemia may promote the transfer of cytochrome from mitochondria to the cytoplasm [4]. The breakdown of hemoglobin may cause the formation of unstable ferrous ions (Fe^{2+}) [5]. The dysfunction of Na^-K^-ATP pumps in the membrane may also lead to the destruction of lysosomes and the mass production of NADPH after tissue ischemia, and tissue hypoxia may promote the production and secretion of Fe^{2+}. The release of iron ions catalyzes the generation of free radicals while aggravating the incidence of oxidative stress and iron metabolism disorder [6, 7]. Previous studies have also suggested that iron overload can worsen kidney injury and iron chelation and antioxidants attenuate renal IRI [8, 9], providing indirect evidence for iron metabolism dysfunction in renal IRI [10].

In addition to the changes in iron metabolism in the kidneys, changes in iron metabolism indexes were also observed in the serum, liver, spleen, and duodenum in our previous study [2]. Our previous study also demonstrated changes in hepatic hepcidin expression and the level of serum hepcidin. Hepcidin is mainly syn-
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Thesized and secreted by hepatocytes [10], acting as the critical factor in regulating iron homeostasis [11]. An accumulating body of research suggests that hepcidin regulates iron efflux and stabilizes intracellular iron by binding to ferroportin-1 (FPN1), inducing its internalization and degradation, and promoting iron intake by the reticuloendothelial (including the liver and splenic macrophages) to maintain the balance of iron metabolism [12]. In our previous study [2], we found that hepcidin might be involved in maintaining iron homeostasis in renal IRI and the kidneys might protect themselves by regulating iron homeostasis in renal IRI. To further examine the role of hepcidin in renal IRI, we treated rats with exogenous hepcidin and analyzed the changes in iron metabolism indexes and their association with renal function in a well-established animal model of renal IRI.

However, it is uncertain whether hepcidin is involved in the regulation of iron metabolism during renal IRI. The present study investigated the role of hepcidin by using an animal model of renal IRI.

Materials and methods

The animal model

A total of 48 Sprague-Dawley (Sino-British SIPPR/BK Lab. Animal Ltd., Co., Shanghai, China) male rats (weighing 200±20 g) were used in this study. Before the experiment, all rats were allowed free access to a standard diet and water and were subjected to a 12 hour day and 12 hour night at an ambient temperature of 24~26°C with a 50-60% humidity for one week and were cared for in accordance with the National Institute of Health guidelines. This study was approved by the Laboratory Animal Use and Management Committee of Shanghai Jiao Tong University School of Medicine.

Experimental design

Based on previous research results [2], the drug intervention was performed before establishing the animal models. The rats were randomly divided into two groups: (1) the hepcidin group (n=24): the rats were injected with hepcidin (0.5 mg/kg, intraperitoneally; Peptides International, USA) twice at 24 hours and 16 hours before the model establishment; and (2) the control group (n=24): the rats were injected with normal saline (5.0 ml/kg, intraperitoneally) twice at 24 hours and 16 hours before the model establishment. According to the differences in modelling methods (renal pedicle occlusion for 45 minutes or not) and renal reperfusion time, the rats were then respectively divided into four subgroups (sham, IRI 4 h, IRI 12 h, and IRI 24 h, n=6/each).

Renal ischemia/reperfusion (I/R) was performed in the Sprague-Dawley rats as previously described [13]. The rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital at a dose of 40 mg/kg (Sigma, St. Louis, USA). The right kidney was then removed from the rats and a non-traumatic vascular clamp (FST, Essen, Germany) was applied to the left renal pedicle for 45 minutes. Then, the clamp was removed for renal reperfusion before the wounds were closed. The rats in the sham group underwent a right kidney excision without the clamping of the left renal pedicles.

All rats survived during the experiment. Samples were collected at 4 hours, 12 hours, and 24 hours after the renal reperfusion in the IRI groups and immediately after a 45-minute exposure of the left kidney in the sham group. The blood samples were collected to measure the concentration of serum creatinine (SCr), blood urea nitrogen (BUN), serum iron (SI), serum ferritin (SF), and hepcidin. Simultaneously, the liver and kidneys were harvested for the histological examination, real-time PCR studies, and iron content analysis while the spleen and duodenum were harvested for the iron content analysis. The experimental design is shown in Figure 1.

The blood biochemical determination

The serum samples were separated by centrifugation at 3000 rpm for 15 minutes, the levels of SCr, BUN, SI, and SF were determined using an automated biochemical analyzer (Beckman Coulter, Inc., CA, USA). The hepcidin levels in the serum samples were quantified using an enzyme-linked immunosorbent assay (ELISA) (Jiancheng Biotechnology, Nanjing, China) according to the manufacturer’s instructions.
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The iron content analysis

The samples of the liver, kidney, spleen, and duodenum were ground with normal saline to make tissue homogenates. After microcentrifugation (Eppendorf, Germany) (2500 g for 10 minutes at 4°C), the supernatants of tissues were collected and protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Nanjing, China). According to the iron content assay kit instructions (Jiancheng Biotechnology, Nanjing, China), the iron contents of the tissue homogenates were determined spectrophotometrically by measuring the presence of iron bipyridine reactive substances. Absorbance was measured using a spectrophotometer (BIO-RAD, USA) at 520 nm. The results were expressed as µmol/g of the protein-based on a prepared standard graph.

The histopathological analysis

After the fixation of the kidney samples that were placed in 10% formalin for 48 hours, the histological paraffin blocks were routinely prepared. A microtome (Leica Rotary; Leica Microsystems GmbH, Wetzlar, Germany) was used to obtain 4-µm-thick sections from the paraffin blocks. The collected sections were stained with hematoxylin-eosin (HE). Then, the sections were examined under 200 × magnification using a light microscope (Leica DM5500B; Leica Microsystems GmbH, Wetzlar, Germany) by a pathologist blinded to the groups, and photos were taken. The tubulointerstitial damage score system was used to analyze the degree of kidney injury in renal IRI [14]. Tubulointerstitial damage was defined as tubular atrophy, tubular ectasia, loss of epithelial cells brush border, and inflammatory cell infiltration. The semi-quantitative pathological scoring method was used to evaluate the degree and range of injury: (1) 0, normal; (2) 1, microlesion (<25%); (3) 2, mild injury (25-50%); (4) 3, moderate injury (50-75%); and (5) 4, severe injury (>75%). The pathological sections were numbered with random numbers, and 10 non-overlapping visions for each section were surveyed by a pathologist.

Real-time PCR

Total RNA was isolated from the liver and kidney samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA). A total of 1 µg of the total RNA was transcribed into cDNA using Prime Script RT master mix (Takara, Japan). All polymerase chain reactions (PCRs) were performed using an ABI ViiA™ 7 Real-Time PCR System (Applied Biosystems, ABI, USA) and SYBR green (Takara, Japan) in a total volume of 20 µL. β-Actin served as an internal control. The following primer sequences were used: hepcidin forward: 5’-TCTCTGGCTTCTCCTCGTGC-3’; hepcidin reverse: 5’-TGTTATCCAGACACAGACCAG-3’; FPN1 forward: 5’-TTGCTGTTCTTTGACCTGTTGT-3’; FPN1 reverse: 5’-GAGGAGCTGTTCGAGATGGTG-3’; β-actin forward: 5’-AGGATG-CAGAAGGAGATTACTGC-3’; β-actin reverse: 5’-AAAACC-CAGCTCGATACGATG-3’.

Western blotting

Proteins from liver and kidney tissues were extracted using RIPA buffer with the inhibitor phenylmethanesulfonyl fluoride (Beyotime, Nanjing, China). Protein concentrations were determined using a BCA protein assay kit (Beyotime, Nanjing, China). A total of 40 µg of...

Figure 1. The experimental design. The rats were divided into two groups: the hepcidin-treated group (Hepcidin) and normal saline-treated group (Control). The drug intervention was performed twice (24 hours and 16 hours) before establishing the animal models. According to the difference of the modelling methods (renal pedicle occlusion for 45 minutes or not) and renal reperfusion time, the rats were divided into four subgroups (sham, IRI 4 h, IRI 12 h and IRI 24 h). NS: normal saline; IRI: ischemia/reperfusion injury.
each protein was loaded into 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Billerica, USA). Then, the membranes were blocked for two hours with 5% skim milk in TBST buffer. The blocked membranes were incubated with rabbit anti-hepcidin (1:500 dilution; Abcam, Cambridge, UK), rabbit anti-FPN1 (1:1000 dilution; Abcam, Cambridge, UK), and rabbit anti-β-actin (1:1000 dilution; Abcam, Cambridge, UK) antibodies at 4°C overnight. After incubation with a secondary antibody conjugated to horseradish peroxidase (1:1000, Santa Cruz Biotechnology, CA, USA) for 1.5 hours at room temperature, proteins were visualized using ECL reagents (Millipore, Billerica, USA).

### Immunohistochemistry

After deparaffinization and rehydration, the paraffin sections of the liver and kidney tissues were incubated with 3% of hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity. Then, the sections were subjected to antigen retrieval by heating the sections in a microwave oven in a 10 mM sodium citrate buffer (pH 6.0). After blocking with 5% normal goat serum in phosphate buffer solution (PBS), the liver tissue sections were incubated with rabbit anti-hepcidin (1:200 dilution; Abcam, Cambridge, UK) and the kidney tissue sections were incubated with rabbit anti-FPN1 (1:400 dilution; Abcam, Cambridge, UK) at 4°C overnight. After washing the samples with PBS, the sections were incubated with secondary antibodies and finally, the sections were incubated with H2O2-DAB. Negative controls were incubated with 3% serum without primary antibodies. The integrated optical densities (IODs) of hepcidin and FPN1 were analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, Maryland, USA).

### Statistical analysis

We used the software program SPSS 19.0 (Armonk, NY: IBM Corp. Chicago, USA) to conduct the statistical analysis. Continuous variables were expressed as mean ± SD, and discontinuous variables were expressed as a percentage (%). For multiple comparisons, each value was compared by a one-way ANOVA following a Dunnett test when each datum conformed to a normal distribution while the non-normally distributed continuous data were compared using non-parametric tests. The counting data were tested by a chi-square test. A value of P<0.05 was considered statistically significant.

### Results

#### The histological and renal function analyses

When compared with the sham group, the SCr and BUN concentrations in the IRI groups were significantly elevated (P<0.05). Compared with the control group, the SCr and BUN concentration in the hepcidin group significantly declined at 12 hours and 24 hours after the renal ischemia/reperfusion (I/R) (P<0.05). There was no significant difference in the SCr or BUN level between the hepcidin group and the control group in the rats that were subjected to the sham procedure or four hours of reperfusion (Figure 2A and 2B).

Renal IRI was confirmed by analyzing the pathological changes of renal tissues and renal function after renal reperfusion. The analysis of the routinely HE-stained kidney tissue sections revealed varying degrees of renal pathological changes in the IRI groups, including the loss of brush borders, vacuolar degeneration, and necrosis in the epithelial cells while the sham group displayed normal histology (Figure 3A).
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Also, the tubulointerstitial damage scores in the hepcidin group were much lower compared with the control group at 12 hours and 24 hours after renal reperfusion (\(P<0.05\)) (Figure 3B).

**The iron metabolism indexes in the serum**

When compared with the sham group, the SI concentrations in the IRI groups were significantly elevated at 4 hours and 12 hours (\(P<0.05\)). Compared with the control group, the level of SI significantly declined in the IRI 4 h and IRI 12 h groups within the hepcidin group (\(P<0.05\)) (Figure 4A).

As shown in Figure 4B, the level of SF at 4 hours and 12 hours was significantly elevated when compared with the sham group and the hepcidin treatment significantly improved the elevated SF (\(P<0.05\)). The SF concentrations in the IRI groups at 24 hours recovered to the levels when compared with the sham group.

**The iron content in the tissues**

We assessed the renal iron content to analyze iron metabolism in the kidneys after renal reperfusion. Figure 5A shows that the renal iron content was significantly elevated at 4 hours and 12 hours after I/R compared with the sham group. The iron content in the kidneys was significantly elevated at 24 hours after renal reperfusion (\(P<0.05\)).
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Figure 5. The effects of hepcidin on the iron content in the tissues. A. The renal iron content was significantly elevated at 4 hours and 12 hours after renal reperfusion, and it was lower in the hepcidin group compared with the control group. B. The hepatic iron content in the hepcidin group showed a certain rise compared to the control group, but there was no statistically significant difference between the two groups. C. The splenic iron content significantly declined at 4 hours and 12 hours after renal reperfusion compared to the sham group, and there was no statistically significant difference between the sham and 24-hour groups. Also, it was elevated in the hepcidin group. D. The duodenal iron content in the hepcidin group showed a statistically significant decline compared to the control group. The data are presented as means ± standard deviation. *: P<0.05 sham group vs. control group, **: P<0.05 sham group vs. hepcidin group, #: P<0.05 hepcidin group vs. control group at the same time point; n=6 in each group. Control: the rats were treated with normal saline. Hepcidin: the rats were treated with hepcidin. IRI: ischemia/reperfusion injury.

The expression levels of hepcidin and FPN1

When compared with the control group, the hepatic hepcidin mRNA levels in the hepcidin group were significantly decreased (P<0.05). However, when compared to the sham group, the hepatic hepcidin mRNA levels at 4 hours, 12 hours, and 24 hours were significantly increased (P<0.05) (Figure 6A).

The renal FPN1 mRNA expression was significantly decreased at 12 hours and 24 hours after reperfusion in both the hepcidin group and the control group (P<0.05). However, there was no significant difference in the renal FPN1 mRNA between the two groups (Figure 6B).

We further confirmed the increase in hepcidin protein levels in the liver and the reduction in FPN1 protein levels in the kidneys after reperfusion via western blot analysis. Hepcidin protein expression were significantly increased at 12 hours and 24 hours after reperfusion in both the hepcidin group and the control group (P<0.05). However, the expression level of hepatic hepcidin protein showed a significant decrease in the hepcidin group compared with the sham group (P<0.05). Compared with the control group, the splenic iron content in the hepcidin-treated rats showed an obvious elevation (P<0.05) (Figure 5C).

In contrast to the change in the splenic iron content in this study, the duodenal iron content in the hepcidin group declined compared to the control group (P<0.05). The duodenal iron content showed no obvious change in the early stage after reperfusion, but it was significantly decreased at 12 hours and 24 hours compared to the content in the sham group (P<0.05) (Figure 5D).

hours and 12 hours after I/R compared with the sham group (P<0.05). The renal iron content in the hepcidin group was also lower than in the control group at 4 hours and 12 hours after renal I/R (P<0.05).

We observed a specific rise in the hepatic iron content in the hepcidin group in comparison to the control group but there was no significant difference between the two groups. The iron content also showed a decrease after I/R but was not significantly different when compared to the content in the sham group after reperfusion (Figure 5B).

In both the hepcidin group and control group, the splenic iron content significantly declined at 4 hours and 12 hours after renal I/R compared to the sham group (P<0.05). Compared with the control group, the renal iron content in the hepcidin-treat-
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Figure 6. The effects of hepcidin on hepatic hepcidin expression and renal FPN1 expression. A. Hepatic hepcidin mRNA level was significantly increased after renal reperfusion compared with the sham group, and the hepatic hepcidin mRNA showed a significant decreased in the hepcidin group compared with the control group. B. Renal FPN1 mRNA expression levels were decreased at 12 hours and 24 hours after reperfusion both in the hepcidin group and the control group, however, there was no statistically significant difference between those two groups. C. Hepatic hepcidin protein expression showed a significant decreased in the hepcidin group compared with the control group. D. Renal FPN1 protein expression decreased significantly in the hepcidin group compared with the control group. The data are presented as means ± standard deviation. *: P<0.05 sham group vs. control group, **: P<0.05 sham group vs. hepcidin group, #: P<0.05 hepcidin group vs. control group at the same time point; n=6 in each group. Control: the rats were treated with normal saline. Hepcidin: the rats were treated with hepcidin. IRI: ischemia/reperfusion injury. FPN1: ferroportin-1.

Discussion

The outcomes of this study indicate that the levels of serum creatinine, blood urea nitrogen and serum iron, the renal iron content, and the kidney injury score were significantly decreased in the hepcidin group. Moreover, the expression level of the FPN1 protein was significantly decreased in the hepcidin group compared with the control group (P<0.05). Furthermore, the expression level of the FPN1 protein was significantly decreased in the hepcidin group compared with the control group (P<0.05) (Figure 8).

The hepcidin expression in the liver significantly increased at 12 hours and 24 hours after renal reperfusion in both the hepcidin group and the control group (P<0.05) (Figure 6C). On the contrary, renal FPN1 protein expression decreased significantly in the hepcidin group compared with the control group (P<0.05) (Figure 6D), and which was not parallel to the expression of FPN1 mRNA.

Immunohistochemical analyses of hepcidin and FPN1

Iron is one of the most important factors resulting in apoptosis in renal IRI, and a systemic iron metabolism disorder can aggravate kidney injury. Hepcidin is a key regulator of iron absorption and homeostasis in mammals and is a critical factor in regulating iron homeostasis. Previous research has confirmed that hepcidin regulates iron efflux and stabilizes intracellular iron by binding to FPN1 and promotes iron intake by the reticuloendothelial (including the liver and splenic macrophages) to maintain the balance of iron metabolism. Hepcidin can also inhibit iron absorption from food in duodenal epithelial cells and can equally reduce the iron transfer from duodenal epithelial cells to blood circulation [15, 16]. The regulatory effect of hepcidin
was observed in the present study. The exogenous hepcidin promoted iron absorption by the spleen and suppressed iron absorption and transfer in the duodenum, resulting in a lower level of SI and renal iron content. These effects may contribute to alleviating the iron overload-related kidney injury in renal IRI. Simultaneously, the downregulation by hepcidin of renal FPN1 protein observed in the present study may be beneficial to the stability of intracellular iron and reduce the occurrence of iron-related tissue injury while protecting renal function during renal IRI as a result [17].

A reduction in splenic iron content was also observed in the present study at the early stage of renal IRI, which implies iron release from the spleen at the early stage of renal IRI, irrespective of hepcidin intervention. The reduction in iron content in the spleen may result in the rapid increase in SI at the early stage of renal IRI. However, hepcidin-treated rats showed relative mitigation of splenic iron release to some extent during renal IRI [18]. However, the reason that IRI promoted iron release from the spleen and/or the liver is unclear. Some researchers have suggested that renal hypoxia during renal IRI may stimulate the erythropoietic response, which will further induce iron release from macrophages. The exact mechanism for this requires further study.

Figure 7. Immunohistochemical analyses of hepcidin. A. Immunohistochemical staining of hepcidin protein in the liver. B. Analysis by calculating the integrated optical density (IOD) of immunohistochemical staining of hepcidin protein. The hepatic hepcidin expression were significantly increased at 12 hours and 24 hours after renal reperfusion both in the hepcidin group and the control group. However, the hepatic hepcidin expression was significantly decreased in the hepcidin group compared with the control group. Original magnification, ×400. The data are presented as means ± standard deviation. *: P<0.05 sham group vs. control group, **: P<0.05 sham group vs. hepcidin group, #: P<0.05 hepcidin group vs. control group at the same time point; n=6 in each group. Control: the rats were treated with normal saline. Hepcidin: the rats were treated with hepcidin. IRI: ischemia/reperfusion injury. FPN1: ferroportin-1.

Figure 8. Immunohistochemical analyses of FPN1. A. Immunohistochemical staining of FPN1 protein in the kidneys. B. Analysis by calculating the integrated optical density (IOD) of immunohistochemical staining of FPN1 protein. Compared to the sham group, the FPN1 protein expression level showed a significant decrease after reperfusion, and it was significantly decreased in the hepcidin group compared with the control group. Original magnification, ×200. The data are presented as means ± standard deviation. *: P<0.05 sham group vs. control group, **: P<0.05 sham group vs. hepcidin group, #: P<0.05 hepcidin group vs. control group at the same time point; n=6 in each group. Control: the rats were treated with normal saline. Hepcidin: the rats were treated with hepcidin. IRI: ischemia/reperfusion injury.
In contrast to the significant change in splenic iron content during renal IRI, the change of iron content in the liver was not obvious. Previous studies also did not observe increases in hepatic iron in hepcidin-treated mice and an obvious increase in hepatic iron content by intraperitoneal injection of hepcidin 24 hours after ischemia in mice. However, Scindia et al. [1] observed a similar phenomenon in their study. Some researchers consider hepatocytes to be relatively weak in response to hepcidin and their sensitivity to hepcidin was lower than that of splenic macrophages in previous analyses [19]. However, the abovementioned studies used brief and low doses of hepcidin intervention. The activity of exogenous hepcidin on hepatic iron absorption requires further research with longer treatment times and higher doses of hepcidin intervention.

In our study, the serum hepcidin concentrations were observed to be significantly elevated in hepcidin-treated rats. However, the expression levels of hepatic hepcidin mRNA and protein showed the opposite pattern, which was observed to be significantly lower in hepcidin-treated rats during renal IRI. This was consistent with the findings from previous studies, revealing that exogenous hepcidin may inhibit the synthesis of endogenous hepcidin in the liver. We also found that, accompanied by an increase in SI during renal IRI, the expression levels of hepatic hepcidin mRNA and protein showed an increase in hepcidin-treated rats, indicating that the elevated SI may stimulate the expression of hepcidin in the liver [20], which was not affected by the serum hepcidin concentration during renal IRI. As a result, the elevated SI stimulated the expression of hepatic hepcidin, which further regulates iron homeostasis during renal IRI.

Although the expression of FPN1 mRNA in the kidneys decreased at 12 hours and 24 hours after reperfusion, we did not observe any difference in it between the hepcidin group and the control group, which showed a non-parallel decline with FPN1 protein expression after reperfusion. The difference between the expression of FPN1 protein and mRNA indicates that other factors may lead to the downregulation of FPN1 mRNA expression during renal IRI and the exact mechanism needs further study.

Conclusion
Hepcidin can promote iron intake in the spleen, reduce iron absorption and the exportation in the duodenum, inhibit iron release by the spleen, and alleviate the degree of SI. Changes in iron metabolism may be associated with the remission of kidney injury. Therefore, iron metabolism regulation by hepcidin may have a protective effect on the kidneys in renal IRI.

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Disclosure of conflict of interest
None.

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