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Hepcidin as a Biomarker of Impaired Renal Function in Rat Models for Chronic Allograft Nephropathy

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Data Interpretation D
Manuscript Preparation E
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Background: To explore the use of hepcidin as a marker of impaired renal function in a rat model for chronic allograft nephropathy (CAN).

Material/Methods: Twenty-four models were developed and 20 models were included in this study, using Fisher (F344) rats (donors) and Lewis rats (recipients). Renal function tests were performed preoperatively and postoperatively. Hepcidin, interleukin-6 (IL-6), and erythropoietin levels in serum and urine were measured by enzyme-linked immunosorbent assay (ELISA). To observe pathological changes in the kidneys, 10 rats each were sacrificed at 2 months and 4 months after surgery.

Results: After transplantation, the serum hepcidin and IL-6 levels increased, while urine hepcidin levels decreased. Erythropoietin levels showed a similar trend; all $P<0.05$. Serum creatinine (SCr) and blood urea nitrogen significantly increased post-operatively, with SCr positively correlating with serum hepcidin. Serum hepcidin positively correlated with IL-6 and negatively correlated with EPO. Histopathological results were consistent with CAN, after transplantation.

Conclusions: Hepcidin may be considered as a potential marker of impaired renal function.

MeSH Keywords: AIDS-Associated Nephropathy • Hepcidins • Kidney Transplantation • Rats, Inbred ACI

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Background

In recent years, there has been a continuous improvement in the development of new immunosuppressive reagents and therapies leading to a significantly reduced rejection rate after renal transplantation. However, the long-term survival rate still remains a concern. The loss of renal function at the late stage of transplantation is mainly caused by chronic allograft nephropathy (CAN), accounting for approximately 40% of the functional loss. The pathological mechanisms, prevention, and treatment of CAN are currently the primary focus of all transplantation centers. Rat models are often used to study CAN [1]. Recent research has identified hepcidin as an important hormonal regulator in iron homeostasis that is associated with the changes in the levels of numerous hypoxia-inducible factors and inflammatory mediators [2]. Recent reports indicate that hepcidin could be a marker for impaired renal function [3]. Our study is the first to investigate the expression and regulation of hepcidin in CAN.

Material and Methods

Materials

Fisher and Lewis rats weighing 200–250 g were provided by the Shanghai Laboratory Animal Center and raised in the pathogen-free animal facility. Surgical microscope (XTS-6A) was purchased from Zhongtian Optical Instrument Factory (Zhengjiang, Jiangsu Province). Microscopic devices were procured from the Shanghai Surgical Instrument Factory. A traumatic sutures for blood vessels (8-0 and 9-0), intravenous infusion pump, disposable intravenous catheters, and hypertonic citrate adenine (HC-A) solution were provided by the Shanghai Blood Transfusion Center. Cyclosporine A (CsA), sodium pentobarbital, and 125 U/mL heparin saline (4°C) were purchased from Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd.

Surgery protocols

Pre-operative preparations

F344 rats were used as donors and Lewis rats as recipients. Rats were fasted for 12 h (fed only water) before the surgery and perfused with CsA solution into stomach. Surgical instruments were sterilized using 2% Glutaraldehyde. HC-A solution was frozen into ice.

Obtaining the kidney from the donor

The mount of 1.5% (1.5 g/100 mL) sodium pentobarbital was administered to anesthetize the donor rat at a dose of 20 mL/kg. Thereafter, the anesthetized rat was fixed on the operating table with tapes and sterilized with iodine. An incision was made in the middle of the belly to obtain maximum exposure of the abdomen. After moving the left intestine to the right, it was covered with gauze soaked in saline. Warm normal saline was sprayed from time to time. The aorta and vena cava were completely exposed and isolated. The distal end of the bifurcation of the aorta was tied. The proximal end of the mesenteric artery was isolated up to the opening. Within this frame, the ligature was introduced in the artery and vein (1-0), in order to block the blood flow while performing the kidney perfusion. The vascular sheath was opened and isolated up to about 0.3 cm length from the artery and vein of the left kidney. The proximal end was the opening of vena cava and the distal end was the adrenal vein near the renal hilum. The vessels were ligated and the adrenal vein was disconnected from the reproductive artery and vein. The ureter and bladder were isolated using sharp instruments. The urethra and ureter on the right side were ligated to obtain the intact bladder. The left kidney was separated from the surrounding tissues. An indwelling catheter was placed into the abdominal aorta and the perfusion commenced. In the meantime, the proximal end of the abdominal aorta was tied. A small incision was made on the vena cava as an outflow track and its proximal end was ligated, and perfused with HC-A solution cooled to 0–4°C at a speed of 60 mL/h using the intravenous infusion pump, until the kidney became white and the outflow from renal vein became clear. The total amount of solution used was about 5 mL. After perfusion, the renal artery and vein were disconnected. The donor kidney was preserved with the oval arterial valve and some vena cava in the preservation solution at 0–4°C for 1–1.5 h.

Allograft transplantation

The recipient was anesthetized in the same way as the donor. The abdomen was exposed carefully, while exposing the left kidney. The abdominal aorta and vena cava were isolated (to avoid tying the vessel branches), along with the left renal artery and vein (leaving the adrenal and reproductive vein intact). Blood flow was blocked below the mesenteric artery. The left kidney was removed, while retaining the renal vein near renal hilum (about 0.5 cm). The vessels were ligated for later use. The renal artery was isolated using anastomosis. The ureter was ligated and excised.

The donor kidney was stored on ice to keep it fresh. The blood flow in the left renal vein was blocked using microsurgical vascular clamps. The kidney was rinsed with 4°C sodium heparin saline to remove the retained blood. Using a 3-step method, we re-connected the veins: by suturing the trisection points of the renal veins from donor kidney and the recipient using the 9-0 sutures, and hanged. Arteries were reconnected by end-to-side anastomosis: by blocking the abdominal aorta and

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dissecting the left renal artery. An oval incision was made at the aorta-renal-artery bifurcation that is slightly larger than the artery of the donor kidney. The abdominal aorta was fixed using 9-0 sutures and stitched up to 6-8 times between the fixing points. Before the last stitch of re-connection, the artery or the vein was perfused with 4°C sodium heparin saline. Before placing the kidney in the abdomen, the ice was removed, and the kidney was soaked in warm water to harmonize with the physiological temperature. The vascular clamps on the renal vein and the abdominal aorta were released (the clamps at the bottom were released first), and see whether there was any sign of bleeding from the incision. If bleeding occurred, the incision was gently pressed with swabs for 1–2 min. The transplanted kidney was monitored for quick vascularization with the blood from the renal vein. The renal arterial pulses were clearly seen. The ureteral movement was observed after 2–4 min. An incision was made on the top of the recipient bladder of similar size as the bladder valve of the donor kidney. The urinary tract was reconstructed by reconnecting the donor kidney and the recipient bladder using 2-point method with 9-0 sutures. The incision on the abdomen was stitched up with 4-0 sutures. After the surgery, the recipient rat was kept warm by placing near the heater until it regained consciousness.

The removal of the recipient's right kidney and examination of the transplanted kidney

On Day 10 after operation, an incision was made on the right side of the abdomen. The right renal artery, vein and ureter were ligated, and the right kidney was removed. The transplanted kidney was also examined.

Post-operative treatment

All the animals who underwent transplantation were treated with an intraperitoneal injection of penicillin 200 000 U/d for 3 days starting on the day of transplantation; CsA perfusion through stomach for 10 days at the dose of 10 $mg \cdot kg^{-1} \cdot d^{-1}$.

Sample collection and testing

The blood and urine samples were collected 24 hs before the transplantation, and at 1, 2, and 4 months after the transplantation. Urine samples were collected using the metal metabolic cage and volumes were recorded. Urine samples (10 mL) were centrifuged at 5000 rpm (r=4.45cm) for 10 min. The pellets were removed and supernatants were separated and stored at –80°C. Blood samples were collected from the inferior vena cava of the sacrificed rats by using vacuum tubes and adding EDTA (anti-coagulant). Blood samples (4 m) were centrifuged at 3000 rpm at 4°C for 10 min and the sera were stored at –80°C. Hepcidin expression level in serum and urine samples was measured by competitive ELISA, using the kit from RayBiotech, USA (DRE20097). Experiments were performed in accordance with the manufacturer's instructions. Test range was 5–100 ug/L. Quantification of serum IL-6 and EPO was similar to hepcidin as described above, using kits DRE20064 and DRE. Test ranges are 8–150 ng/L and 1–20 U/L, respectively.

Quantification of biomarkers

SCr and BUN were tested using automated biochemical analyzer.

Renal pathology test

Ten rats were sacrificed at 2 and 4 months after surgery, respectively, to obtain the left kidneys. Part of left kidney was used for pathological test and the rest was stored in liquid nitrogen for testing in the future. The renal tissues were fixed with formaldehyde and embedded in paraffin. Sections (2-μm thick) were stained with hematoxylin, eosin, as well as para-amino salicylic acid, and then examined by optical microscopy. Pathological changes were ranked according to Banff 2009 standards [4,5]. The ranking based on tubular atrophy (ct), interstitial fibrosis (ci), and interstitial inflammation (i) is shown in Table 1.

Statistical analysis

SPSS package 13.0 was used for data analysis. Data were presented as mean ±sd ($\bar{\chi}$±s). The t-test was used to compare the difference between the 2 groups and ANOVA was used to analyze the differences among multiple groups. Pearson correlation analysis was used to assess correlations.

Results

The success rate of transplantation

Among the 30 surgeries, 24 of the whole were successful (survived for at least 1 week after transplantation), yielding a success rate of 80%. The average period of operation was 120±20 min (40±10 min for the harvest of the donor kidney and 80±20 min for kidney transplantation). Twenty cases survived in 1 month after surgery and were included in the following experiments. Figure 1 reflects the operation on renal in the transplantation.

Pathological changes in CAN rat models

Compared to normal kidney, the transplanted ones (at 2 months after surgery) showed no significant changes in terms of morphology and size. Pathology tests indicated mild to moderate level of interstitial fibrosis and infiltration of lymphocytes and

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Table 1. Types of chronic allograft nephropathy and their numerical ranking.

Lesions	Numerical ranking	Pathological characteristics
Tubular atrophy (ct)	0	No tubular atrophy
	1	<25%
	2	26–50%
	3	>50%
Interstitial fibrosis (ci)	0	<5%
	1	6–25%
	2	26–50%
	3	>50%
Interstitial Inflammation (i)	0	Monocyte infiltration <10%
	1	10–25%
	2	26–50%
	3	>50%

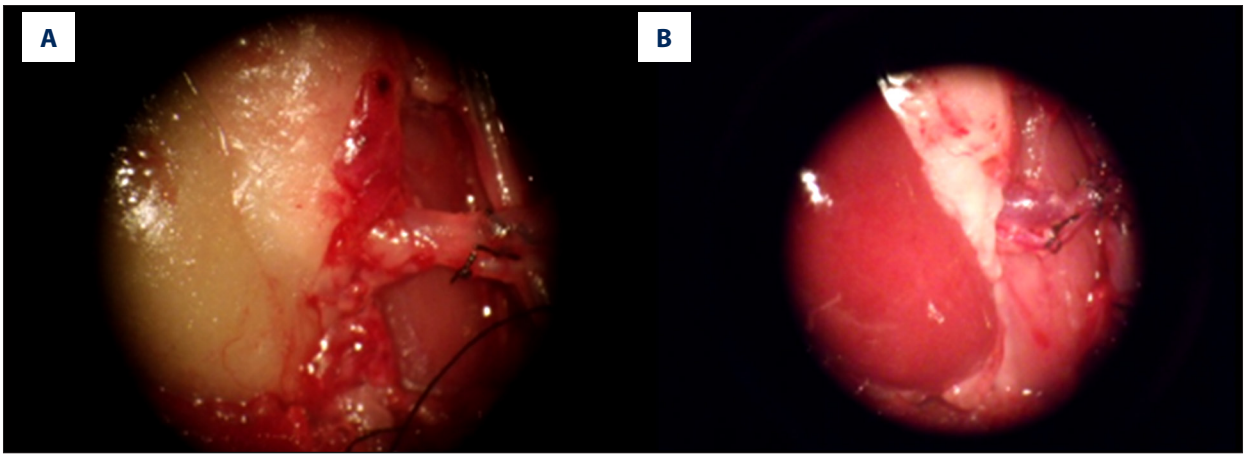

Figure 1. Observations during renal transplantation: (**A**) Renal perfusion *in vivo*; (**B**) Refill of blood after transplantation.

plasma cells. At 4 months after surgery, the transplanted kidney showed a wide range of interstitial fibrosis and severe infiltration of interstitial cells. Thickening, sclerosis and clog of glomerular basement membrane as well as tubular atrophy were observed, which was consistent with the pathological changes in CAN (Figure 2, Table 2). It indicated that the rat models we built accorded with the symptoms of CAN. Therefore, the successfully established CAN rat models were qualified to be tested to investigate the mechanisms of CAN.

The changes of biomarkers and correlation analysis in CAN rat models

The levels of serum Hepcidin showed a continuous growth at 1, 2, and 4 months after surgery compared with that before surgery (all $P<0.05$). On the contrary, the urine hepcidin levels decreased significantly at 1, 2, and 4 months after surgery compared with the initial levels before transplantation (all $P<0.05$). The levels of serum IL-6, BUN, and SCr showed a continuous growth trend with time after surgery, while the growth amplitude of BUN between 2 and 4 months after surgery slowed down. However, the decreased trend of serum EPO levels was found after surgery. More details were shown in Figure 3 and Table 3. In addition, we conducted the correlation analyses to investigate the relationship between serum Hepcidin and co-variables after surgery (Table 4, Figure 4). Serum hepcidin was found positively correlated with IL-6 and SCr, and negatively correlated with EPO. No correlation between serum hepcidin and BUN was revealed.

Discussion

Dennis et al. derived a standard CAN rat model from Fisher 344 and Lewis rats, which is regarded as one of the best models

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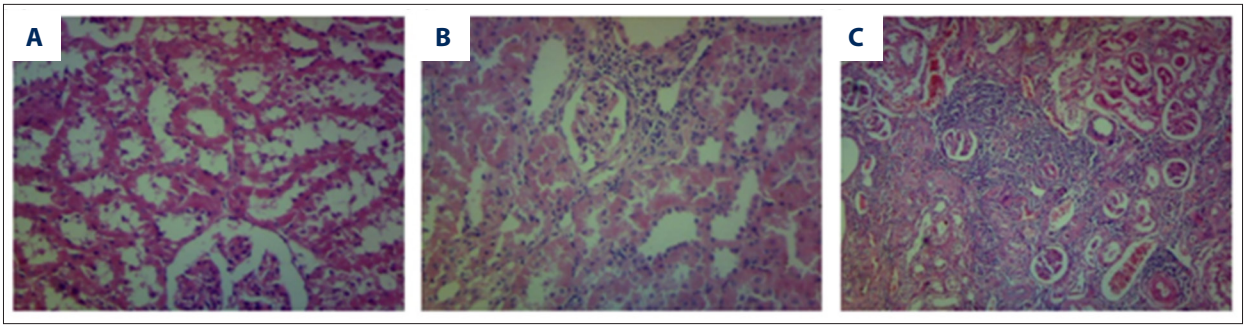

Figure 2. Pathological changes in the transplanted renal tissues (HE ×200) (HE – hematoxylin and eosin stain). (**A**) Normal renal tissues; (**B**) 2 months after transplantation; (**C**) 4 months after transplantation.

Table 2. Pathology ranking after renal graft.

Time	N	Tubular atrophy	Interstitial fibrosis	Interstitial inflammation	Overall ranking
Before transplantation	30	0	0	0	0
2 months after Surgery	10	0.25±0.71	1.63±0.74*	2.13±0.35*	4±1.7*
4 months after Surgery	10	1.88±1.25*,**	2.88±0.35*,**	2.25±0.89*	7±2.2*,**

Compared with pre-transplantation, * $P<0.05$; compared with 2 months after surgery, ** $P<0.05$.

for CAN research [6]. In our study, we performed allograft renal transplantation using F344 rats as the donor and Lewis rats as the recipient. The success rate of transplantation was 80%, which was rather reasonable. The levels of BUN and SCr were detected elevated with the progression of CAN by the functional tests after surgery. The pathological tests showed interstitial fibrosis and severe infiltration of interstitial cells. Meanwhile, thickening, sclerosis and clog of glomerular basement membrane as well as tubular atrophy were also observed in the transplanted kidney. The data suggested that our CAN rat models were well established.

The accuracy in the harvest of the donor kidney, transplantation and post-operative treatment ensures the success and stability of the CAN rat models. Several points in our study are noteworthy:

1. During the process of harvesting the donor kidney, the perfusion was kept at low pressure and uniform speed. We used a minimal amount of perfusion solution. The perfusion with HC-A solution was performed using infusion pump at a speed of 60 mL/h until the kidney became white, and outflow from the renal vein became clear. The amount of perfusion solution was approximately 5 mL. While using syringes for perfusion previously, we observed that it was hard to control the pressure, speed, and amount. If the renal artery is released from clamps and the transplanted kidney not completely refilled, there is a high possibility of the occurrence of vasospasm.
2. An improvement in vascular anastomosis was observed in our study, which guaranteed the success of transplantation. All the surgeries from the harvest of donor kidney to transplantation were carried out with the aid of surgical microscopy. Currently Fisher and Fabre [1] methods are used to reconnect the arteries in CAN rat models, and Fabre and Kamada [7] methods for the vein reconnection. The main drawback of the above methods is the relatively high requirement of experimental devices and surgical skills. In our study, the success rate was quite low and embolism occurred frequently when we originally performed the cuff and 2-point venous anastomosis. Subsequently, we utilized the 3-step method, which is simpler and is reported to have a higher success rate. For the arterial reconnection, we performed a side-to-end anastomosis between renal arterial valve and the abdominal aorta. The oval incision on the abdominal aorta was slightly larger than the artery of the donor kidney, which kept a certain amount of tension at the incision site, and, therefore, prevented embolism and guaranteed sufficient blood supply for the donor kidney. There was prompt recovery when the vascular clamps were released. These methods greatly increased the success rate of transplantation. All the rats survived during the rest of kidney transplantation surgery.
3. The donor kidney was kept cold, for at least 2 h after perfusion. Some researchers have stored the donor kidney for 1 h at a low temperature before the transplantation to ensure the typical CAN pathology changes in the rat models [8].

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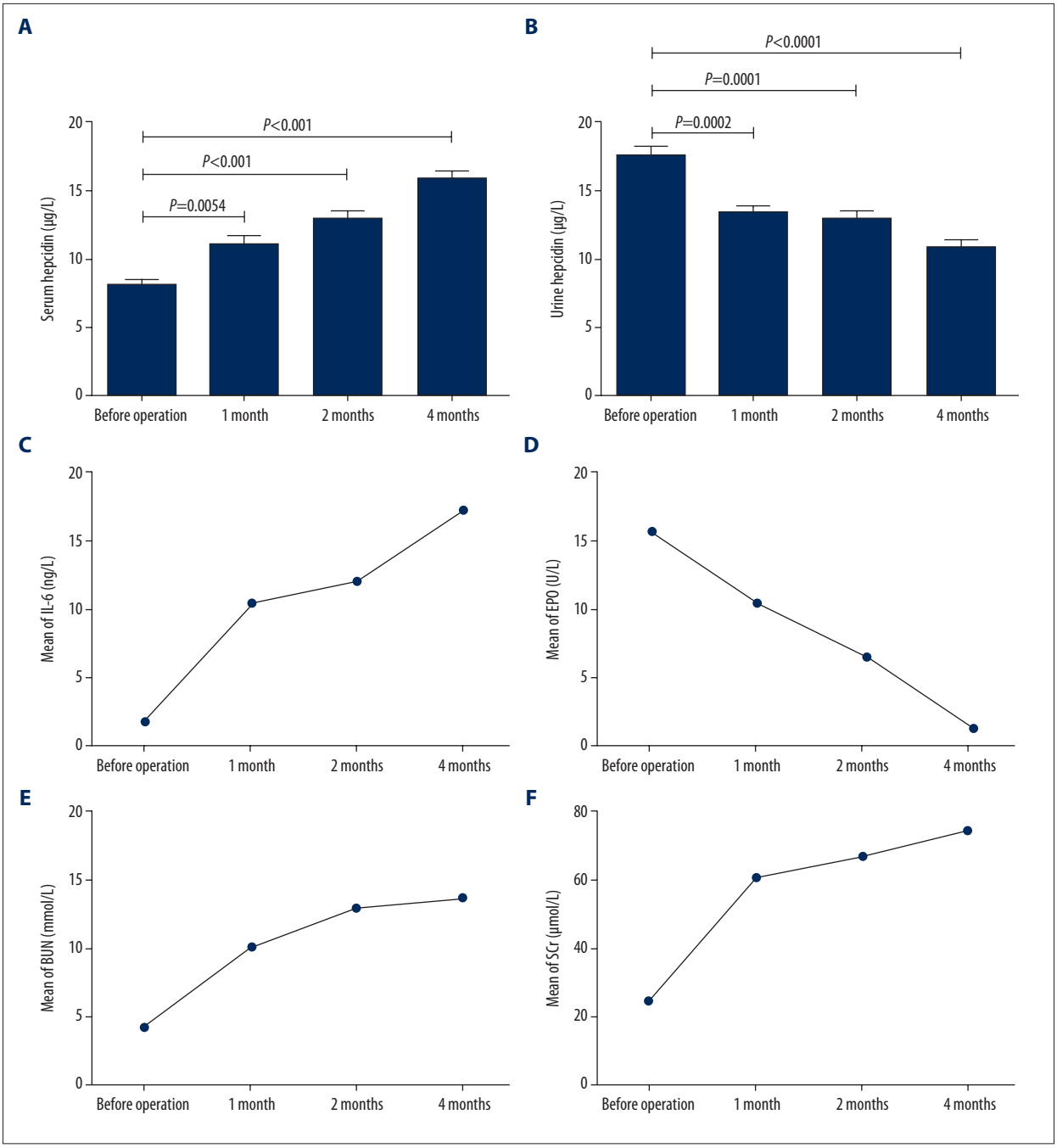

Figure 3. Changes in biomarkers after surgery (IL-6 – interleukin-6; EPO – erythropoietin; BUN – blood urea nitrogen; SCr – serum creatinine). (**A**) Changes in serum hepcidin after operation; (**B**) Changes in urine hepcidin after operation; (**C**) Changes in serum IL-6 after operation; (**D**) Changes in serum EPO after operation; (**E**) Changes in BUN after operation; (**F**) Changes in SCr after operation.

Our preliminary data suggested that 2-h and longer storage at a low temperature led to significant CAN pathological changes, while lesser time had very mild effect.

Renal function tests in regards to the levels of SCr, BUN, and EPO showed that there was a lag between pathological alterations and renal function. There was no significant correlation between the pathological and functional changes. Prowle et al. [9] pointed out that the urine hepcidin levels significantly decreased during renal injury and therefore urine hepcidin could be a sensitive marker for impaired renal functions.

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Table 3. General information from the surgery.

	N	Serum Hepcidin (μg/L)	Urine Hepcidin	IL-6 (ng/L)	EPO (U/L)	BUN (mmol/L)	Cr (umol/L)	Quantification of urinary protein (mg/d)
Before transplantation	30	8.18±1.6	17.52±2.6	2.52±0.41	15.51±0.76	8.2±2.5	38.6±8.9	4.9±1.7
1 Month after surgery	20	10.94±2.1*	13.65±2.8*	15.58±6.94*	10.29±0.58*	12.5±3.1*	65.5±6.1*	35.5±11.7*
2 Month after surgery	20	12.71±2.1*	12.02±1.3*	18.16±7.08*	6.37±0.82*	14.7±2.8*	70.3±8.7*	63.8±52.2*
4 Month after surgery	10	15.81±2.4*	10.13±1.8*	25.71±5.71*	1.23±0.15*	15.2±2.9*	76.1±12.2*	115.4±62.2*

* Comparison between before and after surgery ($P<0.05$). IL-6 – interleukin-6; EPO – erythropoietin; BUN – blood urea nitrogen; Cr – creatinine.

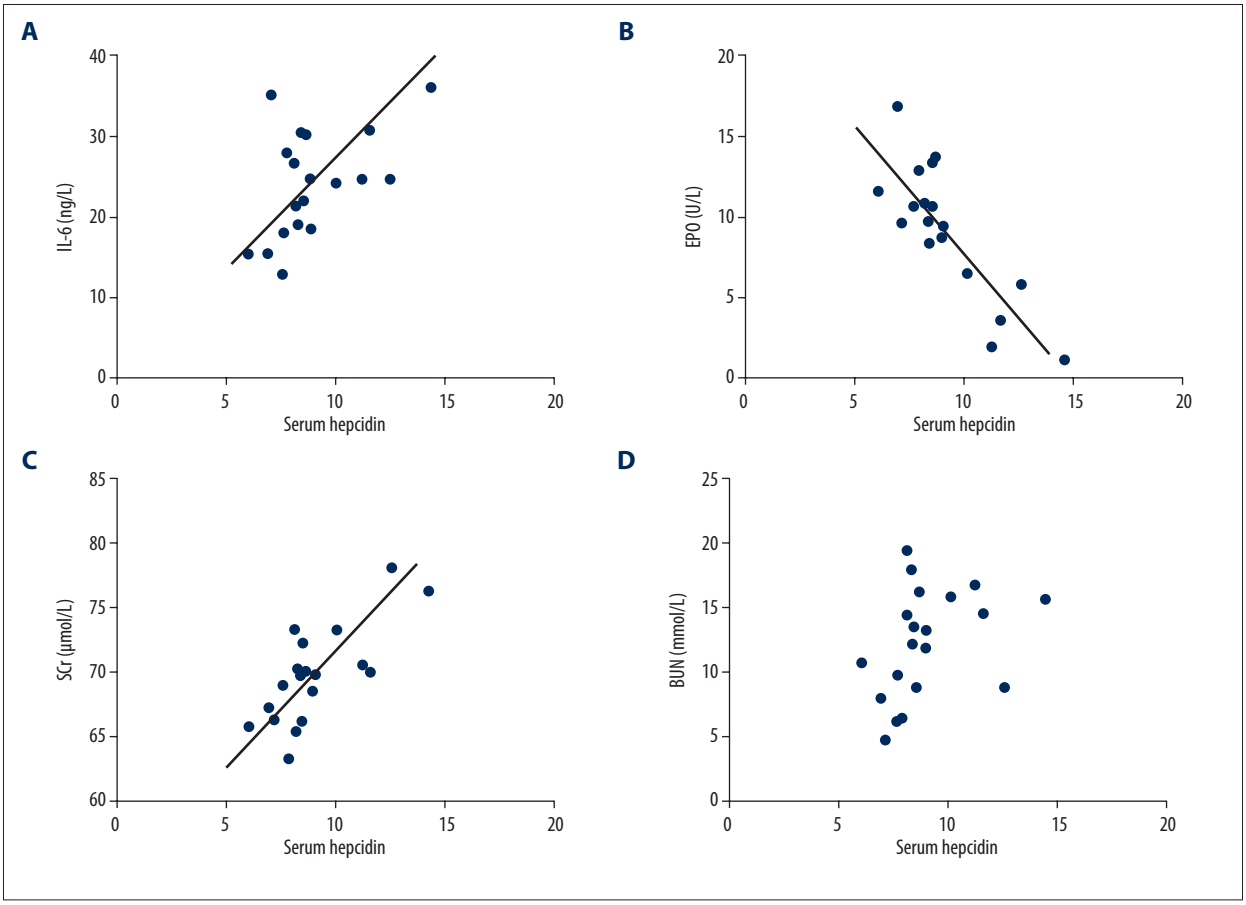

Figure 4. (**A–D**) The correlation analysis between serum hepcidin and co-variables after surgery (IL-6 – interleukin-6; EPO – erythropoietin; BUN – blood urea nitrogen; SCr – serum creatinine).

Hepcidin, synthesized by the hepatocytes, is a newly discovered regulator in iron homeostasis. It plays a central role in iron metabolism by reducing intestinal absorption of exogenous iron as well as the release of endogenous iron from reticuloendothelial macrophages [10]. The expression of hepcidin is closely associated with iron metabolic disorders and chronic diseases resulting from chronic infection and inflammation [11].

By establishing the CAN rat models, we examined the effect on serum and urine hepcidin levels, corresponding inflammatory

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Table 4. The correlation analysis between serum hepcidin and co-variables.

Co-variables	Before transplantation		After surgery	
	Correlation	*P* value	Correlation	*P* value
IL-6	0.012	0.946	0.502	0.024*
EPO	0.291	0.573	–0.838	<0.001*
SCr	0.513	0.102	0.530	0.016*
BUN	0.486	0.147	0.369	0.110

* Serum hepcidin was positively correlated with IL-6 and SCr and negatively correlated with EPO ($P<0.05$). IL-6 – interleukin-6; EPO – erythropoietin; BUN – blood urea nitrogen; SCr – serum creatinine.

responses and biochemical alterations. Statistical analysis of the pathological changes indicated the clinical role of hepcidin in CAN.

Our research further examined the expression of serum and urine hepcidin at different time points after transplantation. Firstly, the results showed that the level of serum hepcidin significantly increased over time since the first month after transplantation ($P<0.05$ for all time points as compared to that before transplantation) and was positively correlated with SCr, indicating that expression of hepcidin was reflected by the progression of inflammation after transplantation, and serum hepcidin was closely associated with renal function. Secondly, it revealed that urine hepcidin was related to the development of CAN. There was a significant difference in urine hepcidin levels before transplantation and at different time points after surgery ($P<0.05$). With the development of CAN, urine hepcidin levels decreased, thus confirming that hepcidin was closely associated with renal function. Another study also reported the relationship between hepcidin and glomerular filtration [12]. Its excretion decreased with impaired renal function; therefore, hepcidin may be used as a convenient marker for testing renal functions. Thirdly, serum hepcidin levels were positively correlated with cytokine IL-6, suggesting that in CAN, IL-6 was possibly a key regulator of hepcidin expression. IL-6 is a major mediator in the inflammatory regulation [13]. IL-6 regulates the expression of hepcidin in serum through activating JAK/STAT pathways, which led to the up-regulation of p-stat3. p-STAT3 inhibited the production of IL-6 induced hepcidin (HAMP), and therefore inhibited the expression of serum hepcidin [14]. The CAN-induced hypoxia and loss of blood resulted in inflammatory responses that regulated the expression of hepcidin via the IL-6-mediated JAK/STAT signaling pathway. Therefore, expression of hepcidin could also reflect the renal pathology changes caused by chronic inflammation. Moreover, EPO is produced and secreted by the kidneys and CAN-induced impairment of renal function can affect the production of EPO. In our research, EPO decreased with the impairment of renal functions, and was negatively correlated with serum hepcidin levels, suggesting that hepcidin is associated with renal disease-induced anemia and impaired renal functions. Dysregulation of iron homeostasis and chronic inflammation largely accounted for the decrease in EPO. Exogenous EPO suppressed the expression of hepcidin. In CAN, EPO affected the expression of hepcidin and caused the alteration in iron metabolism.

Conclusions

The increasing levels of SCr and serum hepcidin were significantly correlated with pathology changes, chronic inflammation, and impairment of renal function with the progression of CAN. Urine hepcidin was closely associated with renal function. Our study demonstrated that during CAN development, loss of blood, hypoxia, and inflammation stimulated the expression of hepcidin, which was correlated with the impairment of renal functions. Therefore, hepcidin can be used as a biomarker for monitoring the renal functions in patients after kidney transplantation, because it acts as a sensitive marker indicating the progression of CAN and inflammatory alterations.

Conflicts of interest

None.

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