Original Article
Comparison of biomarkers in rat renal ischemia-reperfusion injury

Hongying Peng[1*], Yan Mao[2*], Xiaoya Fu[3], Zhipeng Feng[1], Jun Xu[1]

[1]Department of Nephrology, The Affiliated Baiyun Hospital of Guiyang Medical College, Guizhou 550004, China;
[2]Department of Nephrology, Dongfeng Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, China;
[3]Department of Emergency, The First People's Hospital of Guiyang, Guizhou 550004, China. *Co-first authors.

Abstract: To observe the expressions of monocyte chemoattractant protein -I (MCP-I), kidney injury molecule -I (KIM-I) and cystatin C (Cys C) in different periods of rat ischemic acute kidney injury (iAKI). The rat renal ischemia-reperfusion injury (IRI) model was prepared, including the sham-operation (Sham) group and the I/R group. The specimens were collected at different time points after iAKI. The expressions of MCP-1, KIM-1 and Cys C of the I/R group were increased earlier than Scr and Urea (I/R group vs. Sham group; P < 0.01). The serum MCP-1 of the I/R group was earliest increased (MCP-1 vs. KIM-1, Cys C and Scr, P < 0.01). Followed by KIM-1 and Cys C; and in the urine samples, the KIM-1 expression was the most sensitive (KIM-1 vs. MCP-1, Cys C and Scr, P < 0.01). The immunohistochemical results showed the kidney of the Sham group almost had no expression, while that of the I/R group significantly expressed MCP-1, KIM-1 and Cys C (I/R group vs. Sham group; P < 0.01). MCP-1, KIM-1 and Cys C had important predictive values towards AKI, and MCP-1 and KIM-1 were superior to Cys C. Different biomarkers had different sensitivities: MCP-1 was earliest increased in serum while lasted shortly, KIM-1 was earliest increased in urine and kept increasing, thus the detection of urinary KIM-1 might be much more suitable in clinics.

Keywords: Ischemia-reperfusion injury, ischemic acute kidney injury, monocyte chemoattractant protein -I, kidney injury molecule -I, cystatin C

Introduction

The acute kidney injury (AKI) was a new concept proposed by the emergency medical community and international nephrology in recent years [1], which replaced the concept of traditional acute renal failure (ARF), its main purpose, was to advance its clinical diagnosis. Many experts believed that, if the glomerular filtration rate (GFR) was still in the normal phase, it could be identified and performed the early intervenes, which would significantly improve the prognosis of patients [2, 3]. The traditional blood markers of iAKI, such as serum creatinine (Scr), urea and so on, were neither sensitive nor specific towards the iAKI diagnosis [3, 4]. In recent years, through the etiological and pathophysiological researches of iAKI, as well as the genetic and proteomic technologies, a series of new iAKI markers were discovered, including monocyte chemoattractant protein -I (MCP-1) [5], kidney injury molecule -1 (KIM-1) [6, 7], cystatin C (Cys C) [8] and neutrophil gelatinase associated lipid (NAGL) [9], etc. the application of these biomarkers was helpful for the early diagnosis of iAKI, and had great values in determining the lesion severity, improving the outcomes and evaluating the therapeutic effects.

Although domestic and foreign studies had shown that MCP-1, KIM-1 and Cys C could be all used as the early biomarker of iAKI, there was no literature that systematically compared the superiority of these three factors in the serum and urine of IRI model, and there was no comparison among each other of these three factors. This study detected and observed the expressions of MCP-1, KIM-1 and Cys C in serum and urine of rat IRI model, aiming to determine the sensitivity of each factor in serum and urine, and provide the experimental and theoretical basis for the clinical selection of relative marker towards the early diagnosis, prevention and treatment of iAKI.

Materials and methods

Animal model preparation and grouping

48 clean male Wistar rats (180-200 g) were selected, with an average age as 2-3 months old, the animal number was: SCXK (Yu) 2007-0005. The rats were randomly divided into the Sham group (n = 6), and the model group (n = 42, with 6 rats in each sub-reperfusion group (0.5, 1, 2, 4, 6, 12 and 24 h). After intra-peritoneally injected 3% pentobarbital sodium for anesthesia, the rat was cut open the abdomen along the abdominal white line, removed the right kidney, freed the left kidney, then clipped the left renal artery with a non-invasive vascular clamp for 60 min, after that the renal blood supply was recovered. The phenomena observed, namely the graniphyric appearance, as well as the color changes from dark or purple black to bright red, indicated the patho-physiological process of renal ischemia-reperfusion. The rats were killed 0.5, 1, 2, 4, 6, 12 and 24 h after the reperfusion, respectively. The Sham group was only performed anesthesia and laparotomy, while the blood flow was not blocked. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Guiyang Medical College.

Specimen collection and detection

The inferior caval blood was sampled from all rats, after stood for 1-2 h, centrifuged at 3000 r/min for 10 min, and partial supernatant was analyzed by the automatic biochemical analyzer for Scr and Urea, and another partial serum was stored at -80°C for further detection. The urine sample was sampled by bladder puncture, after centrifuged at 3000 r/min for 10 min; the supernatant was kept at -80°C for further detection. The ELISA method was used to detect the concentrations of MCP-1, KIM-1 and Cys C in serum and urine. The rat MCP-1, KIM-1 and Cys C ELISA kits were purchased from R & D Company, USA (repacked by Wuhan Boster Co.).

4°C pre-cold saline was injected from the left ventricle at room temperature to rinse the kidney until the color became white, removed the right kidney, and the moisture was removed with filter paper, the right kidney was then placed into 4% paraformaldehyde for the overnight fixation, followed by the HE staining and immunohistochemical staining.

ELISA

The blank wells (without sample and related enzyme agents, while the rest were the same), standard wells and test sample wells were separately prepared. The experimental procedures referred to the ELISA kit instructions (USA, repacked by Wuhan Boster Co.), added the standards and samples, incubated at 37°C for 30 min, conventionally washed, after that, 50 µl enzyme agents were added into each well and incubated at 37°C for 30 min, then washed five times. 50 µl staining reagent A and reagent B were added, respectively, and stained at 37°C in darkness for 15 min. 50 µl termination solution was added into to stop the reaction. Used the blank well for zeroing, and the OD value of each well was sequentially measured under 450 nm wavelength. The OD values of samples were then input into the equation to calculate the actual concentrations of samples.

HE

The renal tissues were fixed in 4% paraformaldehyde for 24 h, then performed conventional dehydration, wax-dipping, embedding, slicing (3 µm), dewaxing until water, then immersed into hematoxylin for 5 min, and rinsed by water; differentiated in 1% hydrochloric acid alcohol; reduced in 1% ammonia; stained in 0.5% Eosin Y for 3 min; rinsed by water; dehydrated by gradient-concentration alcohol; and hyalinized in xylene for 1 h. Finally, the slices were mounted with neutral gum, and observed under a 400-time magnified optical microscope, each slice was randomly selected 10 visions for the single-blind tubulointerstitial observation.

Immunohistochemisty

Immunohistochemical analysis-SABC method: the renaltissues were prepared paraffin-embedded sections (3 µm), baked at 60°C for 1 h and dewaxed until water, 3% H_2O_2 was used to eliminate the activities of endogenous peroxidases, and microwave-repaired the anti-

Table 1. Concentration of rat Scr and urea ($\bar{x} \pm s$)

Group	n	Scr (μmol/L)	Urea (mmol/L)
Sham	6	40.31±0.56	10.84±2.38
I/R30 min	6	40.43±1.05	10.88±0.17
I/R1 h	6	43.45±3.52	11.01±0.50
I/R2 h	6	46.47±4.47	11.30±2.28
I/R4 h	6	88.15±5.54**	16.40±2.23
I/R6 h	6	118.19±6.81**	18.83±0.62*
I/R12 h	6	132.10±6.70**	29.25±2.17**
I/R24 h	6	153.98±6.27**	35.60±2.69**

Compared with the sham group, *P < 0.05; **P < 0.01.

gens. The normal goat serum blocking group was applied non-specific antigen for 20 min. The primary antibodies (the working concentrations of rabbit anti-rat MCP-1 and KIM-1 were both 1:50, the working concentration of Cys C was 1:70) were dropped inside, incubated at 4°C overnight, the negative control was dropped the same drops of diluted 0.01 M PBS. The secondary antibodies in plus-use SABC kit were added, the negative control used the diluted 0.01 M PBS, and incubated at 37°C for 30 min, PBS washed 5 min × 3 times. Added DAB staining, controlled the reaction time at room temperature for about 3 min, washed with water to terminate the reactions. PBS washed 5 min × 3 times, restained with hematoxylin for 1-2 min, after rinsing, dehydrated with graded alcohol, hyalinized in xylene, and mounted with neutral gum.

Quantitative results of immunohistochemistry

The known positive sample was used as the positive control, 1% BSA was used to replace the primary antibody as the negative control. The positive part under the light microscope exhibited as the brown particles, and the nuclei were of pale blue (the positive situation of MCP-1, KIM-1 and Cys C was the obvious brown granules inside cytoplasm). Finally, the CMI-2011 medical image analysis system was used for the optical microscopic photographing; each slice was randomly selected 10 400-fold magnified visions within the tubulointerstitial area, which did not contain glomeruli and vessels. With the average optical density as the indicator, the Image Analysis-Media Cybernetics-Image Pro Plus 6.0 software was used for the quantitative analysis of immuno-histochemical staining results of each group.

The rabbit anti-rat MCP-1polyclonal antibody and SABC immunohistochemistry kit were purchased from Wuhan Boster Co; the rabbit anti-rat KIM-1 polyclonal antibody was purchased from Shanghai Biococ Biotechnology Lt; the rabbit anti-rat Cys C polyclonal antibody was purchased from Beijing Biosyn Co.

Statistical analysis

The SPSS 17.0 software was used, and the data were expressed as mean ± standard deviation ($\bar{x} \pm s$), the homogeneity of variance was tested firstly, and the variables that met the normal distribution were compared with two independent sample t test, otherwise, the corrected t test was used; the multi-group data were compared with ANOVA, the data with statistical significance by F test were then pairwisely compared with SNK-q test, the comparisons of multiple treatment groups and control groups used the Dunnett-t test. The non-normal distribution data were compared using the Kruskal-Wallis H rank sum test, and the pairwise comparison used the Nemenyi method, with P < 0.05 considered as the statistical difference, and P < 0.01 considered as the statistically significant difference.

Results

Concentration changes of Scr and Urea

Scr and Urea were increased significantly 4 h and 6 h after the reperfusion, and exhibited the statistical significance compared with the Sham group (P < 0.05), with the reperfusion prolongation, the values of Scr and Urea kept rising (**Table 1**).

Serum MCP-1, KIM-1 and Cys C

MCP-1: 30 min after reperfusion, the serum MCP-1 increased, and exhibited the statistically significant difference with the Sham group (*P < 0.05*, **Table 2**), which reached the peak at 6h, then began to decline, but still significantly higher than the Sham group, and the difference was statistically significant when compared with the Sham group (*P < 0.01*, **Table 2**).

KIM-1: 1h after reperfusion, the serum KIM-1 was significantly increased; with the prolonged IR time, the KIM-1 value continued to rise, and exhibited statistically significant difference

Table 2. Concentration of rat serum and urine MCP-1, KIM-1 and Cys C ($\bar{x}\pm s$)

Group	n	Serum			Urine		
		MCP-1 (ng/L)	KIM-1 (ng/L)	Cys C (µg/L)	MCP-1 (ng/L)	KIM-1 (ng/L)	Cys C (µg/L)
Sham	6	148.99±11.94	74.42±3.52	433.28±9.62	286.73±15.67	55.04±5.8	447.72±2.85
I/R30 min	6	276.30±5.32**	82.61±1.79	434.18±10.48	298.81±6.90	70.25±4.22*	448.72±4.33
I/R1 h	6	299.85±13.18**	88.63±0.73**	438.93±9.57	327.44±2.89*	90.71±10.3**	453.97±3.24
I/R2 h	6	306.71±6.78**	95.58±4.74**	452.62±8.82*	343.79±12.05**	110.63±7.97**	464.04±5.42*
I/R4 h	6	342.58±10.61**	104.80±6.50**	469.53±7.59**	403.30±10.67**	110.78±5.73**	483.79±4.12**
I/R6 h	6	377.26±10.53**	107.80±6.48**	481.14±9.30**	407.02±17.15**	121.48±7.04**	501.94±5.29**
I/R12 h	6	361.55±6.82**	112.53±3.62**	547.44±15.04**	399.70±7.69**	124.38±3.85**	576.59±6.57**
I/R24 h	6	342.96±6.46**	126.31±6.20**	622.92±15.79**	388.49±11.79**	142.19±5.61**	658.63±6.30**

Compared with the Sham group, *$P < 0.05$; **$P < 0.01$.

Table 3. Expression of MCP-I KIM-1and Cys C in rat nephric tubule ($\bar{x}\pm s$)

Group	n	MCP-1	KIM-1	Cys C
Sham	6	0.79±0.01	0.19±0.01	0.24±0.02
I/R30 min	6	3.04±0.07**	0.26±0.03*	0.28±0.03
I/R1 h	6	5.29±0.08**	0.35±0.02**	0.34±0.02**
I/R2 h	6	5.41±0.03**	1.18±0.02**	0.61±0.02**
I/R4 h	6	6.32±0.05**	1.75±0.01**	0.65±0.16**
I/R6 h	6	10.42±0.08**	5.20±0.01**	2.03±0.04**
I/R12 h	6	11.84±0.05**	7.81±0.03**	2.72±0.04**
I/R24 h	6	13.86±0.05**	9.09±0.06**	3.87±0.04**

Compared with the sham group, *$P < 0.05$; **$P < 0.01$.

when compared with the Sham group ($P < 0.01$, **Table 2**).

Cys C: The serum Cys C expression was significantly increased 2 h after reperfusion, which had statistically significant difference with the Sham group ($P < 0.05$, **Table 2**). With the IR time prolonged, the Cys C value continued to rise, and the differences were statistically significant when compared with the Sham group ($P < 0.01$, **Table 2**).

Urine MCP-1, KIM-1 and Cys C

MCP-1: 1 h after reperfusion, the urine MCP-1 value was significantly increased, and the difference was statistically significant when compared with the Sham group ($P < 0.01$, **Table 2**), which reached the peak at 6h, then began to decline, but still significantly higher than the Sham group, and the difference was statistically significant when compared with the Sham group ($P < 0.01$, **Table 2**).

*KIM-1:* 30 min after reperfusion, the urine KIM-1 was significantly increased; with the pro-

longed IR time, the KIM-1 value continued to rise, and exhibited statistically significant difference when compared with the Sham group ($P < 0.01$, **Table 2**).

Cys C: The urine Cys C expression was significantly increased 2 h after reperfusion, which had statistically significant difference with the Sham group ($P < 0.05$, **Table 2**). With the IR time prolonged, the Cys C value continued to rise, and the differences were statistically significant when compared with the Sham group ($P < 0.01$, **Table 2**).

Renal histopathological changes

In the early stage of I/R, the model group exhibited pale renal cortex, while the medulla congested, and the color was dark. The glomerulus had the infiltration of inflammatory cells, the tubular epithelial cells proximally and at the cortex-medulla junction mildly swelled, while the medullary tubules did not change significantly. With the reperfusion time prolonged, the tubular damages were increased, the tubule epithelial cells swelled or shrank, ablated, vacuolarly degenerated, and exhibited different degrees of degeneration and necrosis, the nuclei were stained heavily and exposed, partial tubular epithelial cells were brush border-like flat, and partial nuclei ruptured. The cells exhibited the necrosis, the eosinophilic structures disappeared, the tubular lumen stenosis was obstructed or expanded, with the type of protein casts, partial tubules exhibited the ruptured basement membrane, renal interstitial hyperemia, edema and infiltration of large amounts of inflammatory cells; 24 h after reperfusion, the model group showed the high
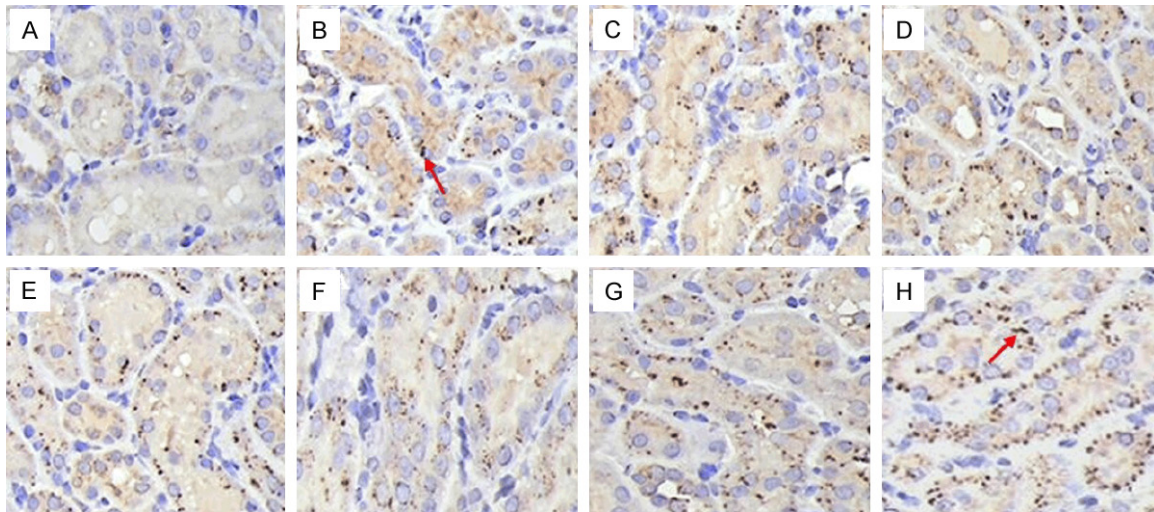
Figure 1. MCP-1 examined in rat nephric tubule by immunohistochemical method. A: Sham; B: I/R 30 min; C: I/R 1 h; D: I/R 2 h; E: I/R 4 h; F: I/R 6 h; G: I/R 12 h; H: I/R 24 h. Scale bar, Magnification, × 400.
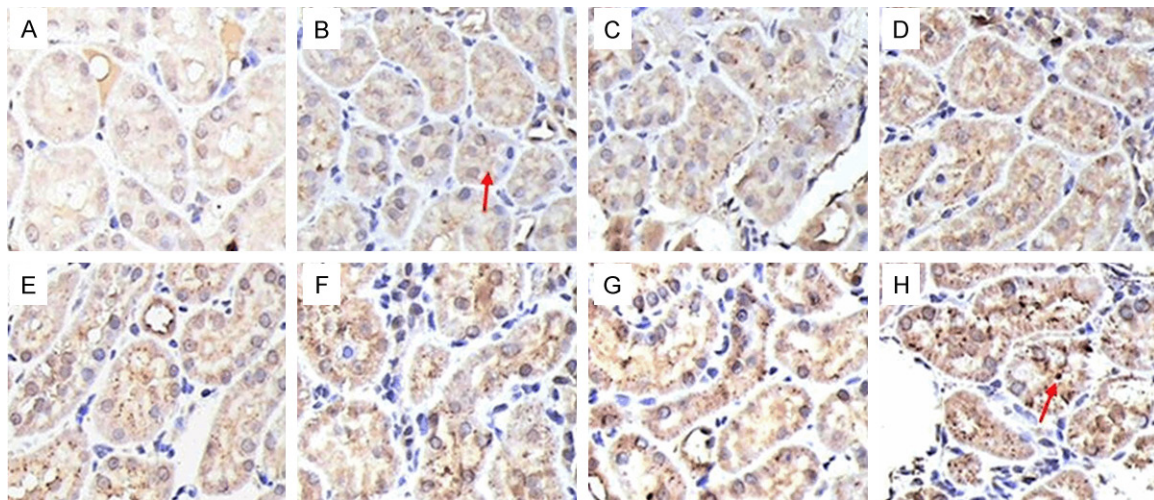
Figure 2. KIM-1 examined in rat nephric tubule by immunohistochemical method. A: Sham; B: I/R 30 min; C: I/R 1 h; D: I/R 2 h; E: I/R 4 h; F: I/R 6 h; G: I/R 12 h; H: I/R 24 h. Scale bar, Magnification, × 400.

degree of renal medullary congestion, karyopyknosis, dissolution and disappearing, most tubular epithelial cells outside the renal medulla exhibited the necrosis and ablation, the distal kidney tubules and the collecting ducts exhibited the cell casts.

Expression of MCP-1

The renal tissues of the Sham group almost had no expression of MCP-1; while 30 min after I/R, the MCP-1 protein was markedly expressed, and exhibited statistically significant difference with the Sham group ($P < 0.01$, **Table 3**), which reached the peak 24 h after reperfusion, and

distributed spottily within the proximal tubular epithelial cells and interstitial substance of exterior medullary mass, while little expressed in the glomerular mesangial areas and capillaries, among the above regions, the protein was expressed by the tubular epithelial cells (**Figure 1**) (the brown particles in **Figure 1** were the positive expression).

Expression of KIM-1

The renal tissues of the Sham group almost had no expression of KIM-1; while 1h after I/R, the KIM-1 protein was markedly expressed, and exhibited statistically significant difference
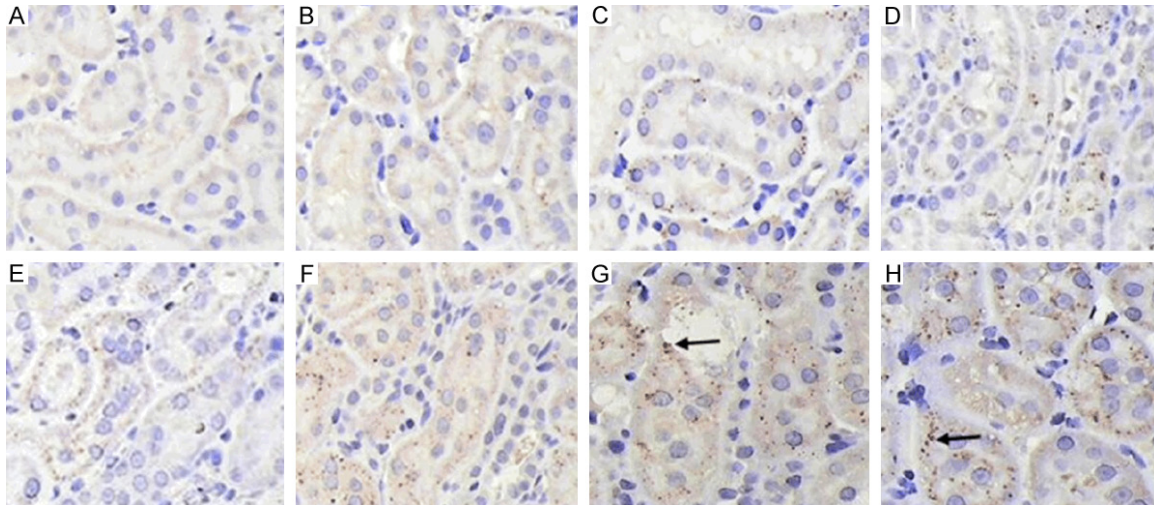
Figure 3. Cys C examined in rat nephric tubule by immunohistochemical method. A: Sham; B: I/R 30 min; C: I/R 1 h; D: I/R 2 h; E: I/R 4 h; F: I/R 6 h; G: I/R 12 h; H: I/R 24 h. Scale bar, Magnification, × 400.

with the Sham group (P < 0.01, **Table 3**), which reached the peak 24 h after reperfusion. The KIM-1 antigen was mainly expressed inside the kidney cortex, proximal tubular epithelial cells and interstitial substance of exterior medullary mass, especially the S3 segment of proximal tubule of exterior medullary mass (this region was very sensitive to ischemia and poisoning injury), the KIM-1 protein was almost not expressed within the internal medullary mass, and could be found the strongest expression at the cortex-medulla joint, the staining was the deepest (**Figure 2**) (the brown particles in **Figure 2** were the positive expressions).

Expression of Cys C

The renal tissues of the Sham group almost had no expression of Cys C; while 1h after I/R, the Cys C protein was markedly expressed, and exhibited statistically significant difference with the Sham group (P < 0.01, **Table 3**), which reached the peak 24 h after reperfusion. The Cys C protein was mainly expressed in the cytoplasm of renal proximal convoluted tubular epithelial cells, little expressed inside the renal collecting duct and kidney glomerulus, while no significantly expressed in the distal convoluted tubule, medullary loop, vessels and renal interstitium (**Figure 3**) (the brown particles in **Figure 3** were the positive expression).

Discussion

The recent studies had found that MCP-1 had different levels of expression in brain, heart,

kidneys and other organs [10-12], it was involved in a variety of diseases, such as tumor development, atherosclerosis and autoimmune diseases [13], and the kidney mesangial cells, renal tubular epithelial cells, endothelial cells and podocytes could secrete MCP-1 under the inflammatory status. Other studies showed that the diabetes could induce the renal MCP-1 concentration and urinary MCP-1 level to increase, which could be used to assess the kidney inflammatory status [14], in recent years, there were many domestic and foreign studies focused on the significance of MCP-1 in AKI, and it expression would be increased in AKI [15]. This study found that: the serum MCP-1 was significantly increased 30 min after I/R (while KIM-1 and Cys C were not significantly increased), indicating that the expression of MCP-1 in serum was the most sensitive; the urine MCP-1 was significantly increased 1h after I/R; MCP-1 in serum and urine reached the peaks 6 h after I/R, then began to decline. Some scholars [16] studied the changes of serum TNF-α and anti-inflammatory cytokines (IL-6) in iAKI, but the mechanism of its dynamics changes was not reported, it was possibly because of the interactions and imbalance between the inflammatory and anti-inflammatory cytokines that determined the dynamic expression of MCP-1, namely in the late stage of I/R, IL-10 could adjust the IRI tissues to produce the preinflammatory cytokines [17]; the animal experiments also confirmed that IL-10 could further reduce the TNF--caused renal injuries in IRI [18]. The mesenchymal stem cells

(MSC) could increase the expressions of IL-10 and bFGF in rat IRI kidney, while reduce the expressions of TNF-α, IL-1β and IFN-γ, thus leading to the expression of MCP-1 decreased in the late stage of IRI.

KIM-1 was a new type of type I transmembrane protein, and its expression would be increased in various reason-caused renal tubular injury [19], the increased expression degree of KIM-1 in renal tubular epithelial cells of ischemia-caused iAKI patients was much higher than other forms of acute and chronic renal injuries [20], this study found that KIM-1 in serum and urine of the model group was earlier than Scr and Urea. In this experiment, KIM-1 in rat urine was significantly increased 30 min after I/R (while MCP-1 and Cys C were no significantly increased at this time), indicating that the KIM-1 expression was the most sensitive in urine; and KIM-1 in serum and urine continued to rise with the extension of reperfusion time. The immunohistochemistry showed that KIM-1 was highly expressed inside the renal tubular epithelial cells of rat AKI model, with a time-dependent manner. When the rat kidney was subjected to ischemia, the peritubular capillaries decreased, and the renal interstitium occurred edema, leading to the cellular ischemia; after injured, the activated tubule cells might secrete large amounts of cytokines, so that the capillary endothelial cells might be further damaged, which might further reduce or block the oxygen supply towards the tubular cells around the damaged regions. In addition, the burden of protein reabsorption by the damaged tubules would be increased, resulting in the activities of lysosomal phagocytosis increased and the oxygen consumption aggravated, which would also contribute the KIM-1 expression to be gradually increased, consistent with previous studies [21].

Cys C had small molecular weight and neutral charge, so it could freely penetrate the glomerular filtration membrane, reabsorb and degrade in the proximal tubule, while not secreted by the renal tubules and not re-absorbed into the bloodstream. Cys C was much more sensitive towards the early and mild changes of renal functions, so it had become a sensitive indicator of early iAKI [22]. This study found that Cys C in serum and urine was significantly increased 2 h after I/R, and its sensitivity was better than Scr and Urea, consistent with other researches. But it was not as sensitive as MCP-1 and KIM-1.

Through this study, we found that MCP-1, KIM-1 and Cys C were increased in serum earlier than Scr and UREA, in summary, these three new markers might be the sensitive and specific biomarkers for the early diagnosis of iAKI. And among these three factors, MCP-1 and KIM-1 were better than Cys C. MCP-1 was the most sensitive in serum; while KIM-1 was the most sensitive in urine. But they also had their own characteristics: for example, MCP-1 might appear in the urine of patients with acute inflammatory lesions, while its specificity was not better than KIM-1; while KIM-1 in urine could be increased 1 h after kidney damage, and its sensitivity was better than MCP-1 and Cys C. As for the expression time, when the expression of MCP-1 reached a certain peak, it began to decline over time, while KIM-I would continue to rise during injury. the urinary KIM-1 was not easy to be interfered by other urinary physical and chemical factors [13], the urinary specimens could be relatively easily collected. Therefore, to detect KIM-1 in urine might be more suitable towards the clinical application.

Disclosure of conflict of interest

None.

Address correspondence to: Hongying Peng, Department of Nephrology, The Affiliated Baiyun Hospital of Guiyang Medical College, No. 108 Corundum Street, Guizhou 550004, China. Tel: +86 851 8441 6010; Fax: +86 851 8441 6879; E-mail: hongyingpengcn@163.com

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