Study on related indexes of the coagulation and fibrinolytic system after renal ischemia reperfusion injury in Wistar rats

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ABSTRACT

الأهداف: عرض تغيرات مؤشرات التخثر والفبرين في مصل الفئران المصابة بإعادة التروية.

الطريقة: أجريت هذه الدراسة في مختبر العلوم العصبية لمستشفى جامعة شانكسي، تايوان، الصين خلال الفترة من سبتمبر واكتوبر 2012م. قمنا بقياس نموذج إعادة الاقفار الكلوي وقياس مصل عينات كل مجموعة لقياس الفيبرينوجين، ومضاد الثرومبين، ومنشط البلازمينوجين، ومنشط مضاد البلازمين، ومنشط البلازمينوجين المانع.

النتائج: بعد إعادة التروية، أظهرت التغيرات قمة مزدوجة للفبرين، انخفض مستوى الفيبرينوجين في ساعتين لأقل مستوى وذلك خلال 12 ساعة ثم رجع إلى قيمتة الأصلية يشكل تدريجي. انخفض مضاد الثرومبين لأقل مستوى خلال 12 ساعة وارتفع إلى أعلى معدل خلال 24 ساعة. بدأ منشط البلازمينوجين المانع بالارتفاع خلال ست ساعات ووصل للقمة خلال 24 ساعة واستردجع قيمته بشكل تدريجي. بينما وصل منشط مضاد البلازمين للقمة خلال ست ساعات وانخفض لأقل قيمه خلال 12 ساعة ثم بدأ استرجاع قيمته خلال 24 ساعة ثم قيمته الطبيعية خلال 36 ساعة. استرجعنا المؤشرات كلها خلال 24 ساعة.

خاتمة: بعد الساعة الثانية من إعادة التروية نظام الكولاجين يصل إلى أعلى قيمة خلال 12 ساعة. كما تم تنشيط كلا من الفيبرينوجين ومضاد الفيبرينوجين في الساعة الثانية. كما وصل نظام الفبرين في الساعة السادسة للقمة خلال 24 ساعة. كما تم استعادة كلا من المعدل الطبيعي للكولاجين والفبرين خلال 24 ساعة.

Objectives: To explore changes in indicators of the coagulation and fibrinolytic system in the serum of rats with renal ischemia reperfusion (IR) injury.

Methods: The study was carried out at the Nephrology Laboratory of the Second Hospital, Shanxi Medical University, Taiyuan, China between September and October 2012. A rat renal IR model was established, and serum samples of each group (8 each) were collected to detect the amounts of fibrinogen (FIB), antithrombin III (AT-III), tissue type plasminogen activator (tPA), plasminogen-antiplasmin complex (PAP), and plasminogen activator inhibitor-1 (PAI-1).

Results: After the start of reperfusion, the changes of FIB exhibited a "double-peak" appearance. The AT-III began to decline at 2 hours to the lowest level at 12 hours, and then gradually recovered. The tPA declined to the lowest level at 12 hours, and then increased to the highest level at 24 hours. The PAI-1 began to increase at 6 hours, and peaked at 24 hours, and then recovered gradually. The PAP peaked at 6 hours, and declined to the lowest level at 12 hours, and began to recover at 24 hours, and was normal at 36 hours. All the above indexes were completely restored at 48 hours.

Conclusion: At 2 hours after reperfusion, the coagulation system were activated, reaching a peak at 12 hours. Fibrinolytic and antifibrinolytic substances were activated at 2 hours, and the fibrinolytic system was inhibited at 6 hours, reaching a peak at 24 hours. Coagulation and fibrinolytic substances were restored to normal at 48 hours.

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Renal ischemia reperfusion (IR) injury refers to the aggravated ischemic injury caused when the blood supply returns to the renal tissue with hypoperfusion after a period of ischemia.[1] An IR injury can lead to vascular endothelial cell damage, and further intravascular thrombus formation. Thrombosis is the result of concurrent effects of tissue injury, platelet activation and coagulation, anticoagulation, and fibrinolysis mechanisms. Under normal conditions, thrombosis and anti-thrombosis are in a dynamic balance in vivo, maintaining the blood flow state in the vessels, and once the balance is destroyed, thrombosis or hemorrhage will be caused.[2] There are complicated interactions including activation and restriction between coagulation and anticoagulation, coagulation and fibrinolysis, fibrinolysis and antifibrinolysis, but the dynamic changes of coagulation and fibrinolysis in the renal IR process are still unclear. Therefore, this paper used a rat IR model to observe the dynamic changes of the related indexes of the coagulation and fibrinolytic system after renal IR, to explore the possible mechanisms of changes in coagulation, anticoagulation, and secondary fibrinolysis function after renal IR injury.

Methods. The study was carried out at the Nephrology Laboratory of the Second Hospital, Shanxi Medical University, Taiyuan, China between September and October 2012. The protocol was carried out in accordance with animal ethical standards. Eighty-eight Wistar rats aged 10 weeks (180-220 g) were randomly divided into 11 groups, with 8 rats in each group, half male, and half female. The Wistar rats were obtained from the Animal Care Center, Shanxi Medical University. They were individually housed in stainless steel cages in a room maintained at 25±2°C with a controlled photoperiod of 12 hours/day. They were fed with commercial pelleted rat chow (Grain silos, Taiyuan, China) and provided with water ad libitum.

The first group was the control group, which was anesthetized before the abdomen was opened, and bilateral renal arteries were isolated and then the abdomen was closed immediately. The second group was the IR 0h group: the bilateral renal arteries were closed with a noninvasive vascular occlusion clamp, and were then opened after renal ischemia of 45 minutes, and the rats were sacrificed. The third group was the IR 0.5h group: the vascular clamp was opened after bilateral renal ischemia of 45 minutes to restore blood perfusion for 0.5 h, and then the rats were sacrificed. The 4th-11th groups were the IR 1h group, IR 2h group, IR 4h group, IR 6h group, IR 12h group, IR 24h group, IR 36h group and IR 48h group, in which, the occlusion of bilateral renal arteries was the same as IR 0h group, and the rats were sacrificed after restoring blood perfusion for 1, 2, 4, 6, 12, 24, 36 and 48 hours respectively, to perform detections as follows.

Serum creatinine and blood urea nitrogen. The serum creatinine (Scr) was determined using the deproteinization method, and blood urea nitrogen (BUN) was determined using the diacetyl monoxime method. Fibrinogen (FIB), antithrombin III (AT-III), tissue type plasminogen activator (tPA), plasminogen-antiplasmin complex (PAP), and plasminogen activator inhibitor-1 (PAI-1) were detected using the ELISA method (R&D Systems, Minneapolis, Minnesota, USA). Blood samples of approximate 5 ml were obtained from the abdominal aorta, and centrifuged at 500 × g for 10 minutes to separate the serum, which was diluted to 1:10 with coating buffer (0.05 mol/L NH_4HCO_3, pH 9.6), with a 100 μL serum sample being added to each hole, and coated at 37°C for 90 minutes. Each hole was washed with 350 μL phosphate buffer (PBS, pH 7.4) 2 times, and blocked with biotin labeled antibody working fluid at 37°C for one hour, and then washed with 350 μL PBS 3 times. One hundred μL avidin-biotin-peroxidase complex (ABC) was added to each hole, and reacted at 37°C for 30 minutes. Then, PBS was used to wash 5 times, and 3,3',5,5'-Tetramethylbenzidine (TMB) was used for coloration. A model type 550 enzyme-labeling measuring instrument (Bio-Rad, Hercules, California, USA) was used for detection at 450 nm and data acquisition.

All data were processed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) 13.0 statistical software, and measurements were presented as mean ± standard deviation. One-way ANOVA was applied for the comparison among multiple samples, while Dunnnet-t or LSD-t test was used for paired comparison. Differences were considered to be statistically significant when $p<0.05$.

Results. The measurements of Scr and BUN in each group are shown in **Table 1** and **Figures 1 & 2**. The Scr (μ mol/L) measurements in the IR 12h group, IR 24h group, and IR 48h group were statistically significantly different to the control group ($p<0.05$). The BUN (mmol/L) measurements in the IR 6h group, IR 12h group, IR 24h group, and IR 48h group were also statistically significantly different to the control group ($p<0.05$). After the start of reperfusion, the changes of FIB exhibited a "double-peak" appearance, which gradually increased from 0h to 6h, and then declined to

Table 1 - Renal functions of rats before and after IR (mean ± SD).

Groups	Scr (μmol/L)	BUN (mmol/L)
Control group	111.00±20.41	6.512±0.009
IR 0h group	118.02±26.99	6.485±0.030
IR 6h group	226.58±42.20	11.213±0.009#
IR 12h group	384.11±12.36#	16.185±0.025#
IR 24h group	624.24±27.68#	27.658±0.024#
IR 48h group	515.12±14.63#	25.467±0.049#

IR - ischemia reperfusion, Scr - serum creatinine, BUN - blood urea nitrogen, #Compared with control group $p<0.05$

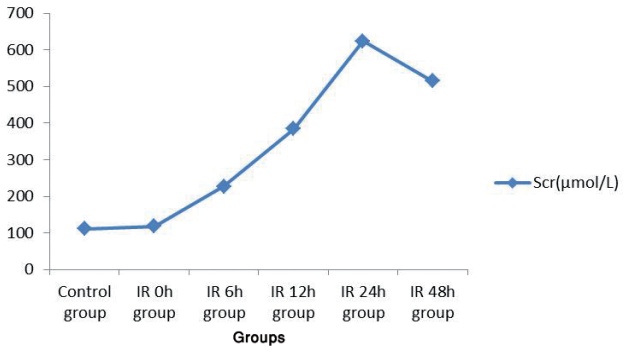

Figure 1 - The contents of serum creatinine (Scr) in the serum of rats before and after ischemia reperfusion (IR).

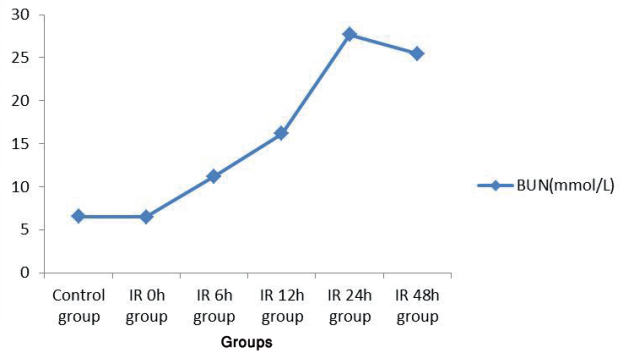

Figure 2 - The contents of blood urea nitrogen (BUN) in the serum of rats before and after ischemia reperfusion (IR).

Table 2 - The contents of FIB and AT-III in serums of rats before and after IR (mean ± SD).

Groups	FIB (mg/L)	AT-III (mg/L)
Control group	2336.17±96.08	7.732±0.053
IR 0h group	2433.33±91.24	7.607±0.372
IR 0.5h group	2583.67±126.63#*	7.248±0.066#*
IR 1h group	2656.50±76.93#	7.167±0.047#
IR 2h group	2684.50±82.09#	6.732±0.014#*
IR 4h group	2757.67±90.01#	6.453±0.029#*
IR 6h group	2821.00±93.73#	5.658±0.041#*
IR 12h group	2003.33±64.45#*	5.187±0.016#*
IR 24h group	3674.00±205.25#	6.315±0.022#*
IR 36h group	2924.33±119.67#*	7.525±0.438*
IR 48h group	2349.83±154.64*	7.713±0.044

IR - ischemia reperfusion, FIB - fibrinogen, AT-III - antithrombin III, #Compared with control group $p<0.05$, *Compared with the previous time point $p<0.05$

Table 3 - The contents of tPA, PAI-1, and PAP in serums of rats before and after IR (mean ± SD).

Groups	tPA (ng/L)	PAI-1 (AU/ml)	PAP (mg/L)
Control group	0.468±0.004	0.272±0.004	8.218±0.013
IR 0h group	0.430±0.014#	0.273±0.010	8.211±0.030
IR 0.5h group	0.401±0.004#*	0.275±0.005	8.228±0.024
IR 1h group	0.433±0.013#*	0.288±0.009#	8.268±0.015
IR 2h group	0.340±0.009#*	0.373±0.005#*	9.418±0.004#*
IR 4h group	0.252±0.070#*	0.478±0.021#*	10.200±0.033#*
IR 6h group	0.188±0.004#*	0.587±0.016#*	10.670±0.022#*
IR 12h group	0.160±0.008#*	0.693±0.014#*	7.421±0.112#*
IR 24h group	0.742±0.007#*	0.760±0.023#*	7.770±0.058#*
IR 36h group	0.418±0.007#*	0.550±0.017#*	8.268±0.043*
IR 48h group	0.455±0.005*	0.283±0.012*	8.280±0.107

IR - ischemia reperfusion, tPA - tissue type plasminogen activator, PAI-1 - plasminogen activator inhibitor-1, PAP - plasminogen-antiplasmin complex, #Compared with control group $p<0.05$, *Compared with the previous time point $p<0.05$

the lowest level at 12h, followed by progressive elevation to the highest level at 24h, and then declined gradually. The AT-III began to decline at 2h to the lowest level at 12h, then gradually recovered. The tPA declined to the lowest level at 12h and then increased to the highest level at 24h. The PAI-1 began to increase at 6h and peaked at 24h, and then recovered gradually. The PAP peaked at 6h, and declined to the lowest level at 12h, and began to recover at 24h, and was normal at 36h (Tables 2, 3, and Figures 3-7).

Discussion. Under normal circumstances, resting endothelial cells have an anticoagulant function, which can regulate thrombinogenesis, fibrinolysis, and platelet activation through a variety of mechanisms. Rapid restoration of blood flow after reperfusion could activate

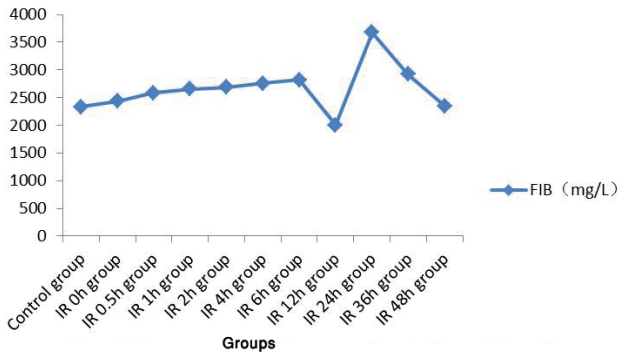

Figure 3 - The contents of fibrinogen (FIB) in the serum of rats before and after ischemia reperfusion (IR).

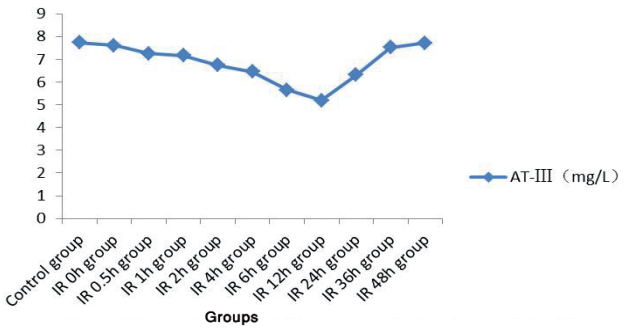

Figure 4 - The contents of antithrombin III (AT-III) in the serum of rats before and after ischemia reperfusion (IR).

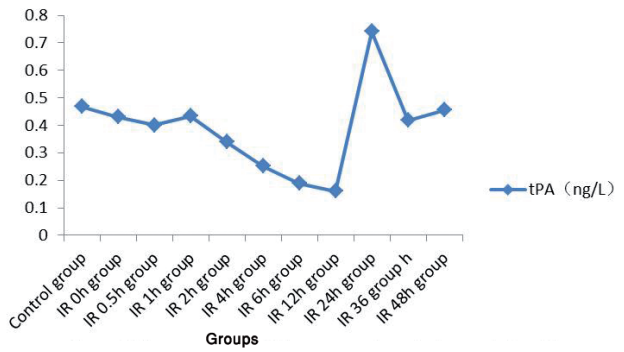

Figure 5 - The contents of tissue type plasminogen activator (tPA) in the serum of rats before and after ischemia reperfusion (IR).

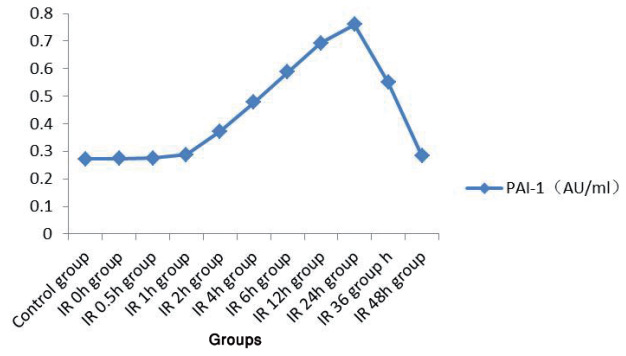

Figure 6 - The contents of plasminogen activator inhibitor-1 (PAI-1) in the serum of rats before and after ischemia reperfusion (IR).

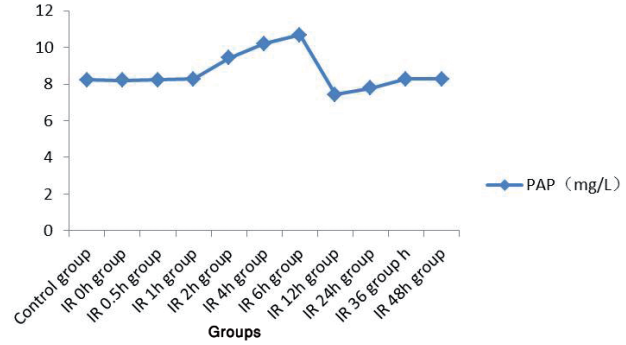

Figure 7 - The contents of plasminogen-antiplasmin complex (PAP) in the serum of rats before and after ischemia reperfusion (IR).

various cell adhesion molecules,[3-5] allowing the increase of adhesion between blood cells and vascular endothelial cells, the adherence and rolling of leukocytes, adhesion and aggregation of platelets, and the activation of vascular procoagulant substances like thrombin. While some anticoagulant components synthesized by injured endothelial cells such as heparin-like glycosaminoglycan can be significantly reduced, so that the plasma FIB is easily attached to the injured vessel surface to be transformed into fibrin, leading to the final coagulation. After hemostasis is complete, the fibrinolytic system is started, and the thrombus is gradually dissolved, thereby ensuring vessels are unobstructed, but also conducive to the repair and regeneration of damaged tissues.[6]

Fibrinogen is a coagulation factor, the highest in plasma, synthesized by hepatocytes, which is a symmetry dimer composed of 3 different polypeptide chains, namely α, β, and γ. The FIB plays an important role in the coagulation mechanism and fibrinolysis process.[7,8] Antithrombin III is derived from liver cells, endothelial cells, and megakaryocytes, belonging to alpha 2 globulins, and it is the main inhibitor of the serine proteases generated in each stage of coagulation, which is one of the main physiological anticoagulant factors, with a very strong anticoagulant effect. The level of AT-III reflects the activity of thrombin, and when coagulation activity of the blood increases, AT-III consumption will reduce, and congenital or acquired AT-III deficiency can cause the risk of developing thrombosis to increase.[9] Tissue type plasminogen activator is an important constituent of the fibrinolysis system, secreted by endothelial cells, which can

transform plasminogen into plasmin with catalytic action, dissolving the thrombus. Plasminogen activator inhibitor-1 is mainly secreted by vascular endothelial cells, and its main function is to prevent plasminogen activated by tPA. Local balance of the fibrinolytic system depends on the concentrations of local tPA and PAI-1,[10] and under normal circumstances, tPA and PAI-1 are in dynamic equilibrium, and capable of forming stable complexes at the ratio of 1:1 in vivo, thereby maintaining the balance of the fibrinolytic system, protecting vascular integrity and unobstructed blood flow.[11,12] If the level of tPA reduces and PAI-1 increases, endogenous fibrinolytic activity will decrease and may lead to thrombosis. Under a physiological or pathological state, free plasmin combines α_2 antiplasmin (α_2-AP) to form a 1:1 compound, to be inactivated as PAP. The generation of plasmin is directly related to the activity of the fibrinolytic system, but approximately 92% plasmin will be inactivated. Due to the difficulty to directly determine plasminogen in plasma, the PAP is taken as the direct marker of plasminogen in vivo. The generation and the increase of PAP are molecular markers of activated fibrinolytic and antifibrinolytic substances, and in the prethrombotic state, the content of PAP increases.

This experiment adopted a renal IR model, and after the start of reperfusion, the serum Scr and BUN increased progressively; reaching a peak at 24h, which suggested the model was successful. This experiment observed that, at 0h after reperfusion, the plasma FIB began to increase, indicating it was a type of stress reaction to the injury; at 2h after reperfusion, PAP increased, and AT-III decreased progressively, which suggested that reperfusion flow caused injury to the vessel wall, vascular endothelial cell inclusions release such as von Willebrand factor, endothelin-1 and tPA, subendothelial components exposure such as collagen, micro fiber and laminin, the coagulation system were activated, and fibrinolysis and antifibrinolytic substances became activated. At 6h after reperfusion, the activity of tPA decreased, and the activity of PAI-1 increased, indicating that there were significant changes in the coagulation and fibrinolytic system, causing a blood hypercoagulable state, and inhibiting fibrinolytic activity at the same time. At 12h after reperfusion, the plasma FIB decreased sharply, indicating that the coagulation mechanism started, and a large number of FIB were transformed into fibrin, resulting in consumptive reduction of FIB, while AT-III also dropped to the lowest level, suggesting the coagulation had reached a peak. At 24h after reperfusion, FIB and tPA increased to the highest level, indicating hyperfibrinolysis at this stage. At 36h after reperfusion, these indexes of the coagulation and fibrinolytic system gradually recovered to normal levels. At 48h after reperfusion, all indexes returned to normal.

In short, there are dynamic changes of coagulation and fibrinolysis in the IR process. Reperfusion induces the injury of vascular endothelial cells, and the coagulation system is activated 2h after reperfusion, reaching a peak at 12h. Fibrinolytic and antifibrinolytic substances are activated at 2h, and fibrinolysis is inhibited at 6h, reaching a peak at 24h, and coagulation and fibrinolysis were restored to normal at 48h.

The limitation of this study is that we did not conduct research on how to reduce renal ischemia reperfusion injury. We will observe the system changes of patients with renal IR injury in future studies, and investigate treatment for renal IR injury according to these changes.

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