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Effect of Complement C1q Expression on Hepatic Ischemia-Reperfusion Injury in Rats*

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Summary: The effect of the complement C1q expression on total hepatic ischemia-reperfusion (I/R) injury in rats was investigated. Sixty healthy male Sprague Dawley (SD) rats weighing 180–200 g were randomly divided into 5 groups: sham-operation group (S group, *n*=12); group of I/R for 1 h (I/R 1 h group, *n*=12); group of I/R for 3 h (I/R 3 h group, *n*=12); group of I/R for 6 h (I/R 6 h group, *n*=12); group of I/R for 24 h (I/R 24 h group, *n*=12). The hepatic I/R model of rats was established, and liver tissues were obtained 1 h, 3 h, 6 h and 24 h after hepatic I/R, respectively. Furthermore, the tissues were stained using hematoxylin-eosin, and the liver injuries of rats were observed using a microscope. The malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in liver tissue were determined. Real-time polymerase chain reaction (PCR) and Western blotting were used to detect the expression levels of C1q mRNA and protein, respectively. As compared with the S group, the histopathological changes in I/R 1 h–24 h groups were gradually aggravated with the extension of I/R time. As compared with the S group, SOD activity and MDA content in the I/R groups were reduced and increased respectively with the extension of I/R time (P<0.01). Furthermore, the C1q expression at mRNA and protein levels in the I/R groups (especially in the I/R 3 h group) was significantly higher than that in the S group (P<0.05). It is suggested that C1q expression may play a principal role in hepatic I/R injury, particularly at the early stage of perfusion.
Key words: hepatic ischemia-reperfusion; complement system; C1q

Hepatic ischemia-reperfusion injury (HIRI) is a common pathophysiological process in clinical surgery. Reduced blood flow in tissue and organ caused by different factors can induce ischemic injury, and the damages of functional metabolism and structure of cells, tissues and organs will be aggregated after the restoration of blood perfusion. For liver transplantation, the early liver graft non-function (accounting for 5% of liver transplantations) and liver dysfunction (accounting for 10%–30% of liver transplantations) are closely related to liver I/R, and HIRI also increases reject reaction of long-term liver transplantation. Hence, HIRI is one of the major issues in critical care medicine, and the prevention of HIRI is important to treat various liver diseases and increase the success rate of liver transplantation. The HIRI degree is tightly related to the extent and time of liver ischemia[1, 2]. Multiple factors are involved in HIRI, and they restrict and promote each other[3, 4].

Complement system is a group of proteins with enzymatic activity in serum and body fluids of humans and vertebrates. The complement system is an important effector and effector amplification system in organism with a variety of biological roles. As an important component of inflammatory response, complement has received more and more concerns. The canonical pathway of complement activation is a series of cascade reactions after combination of specific antigen-antibody complexes with C1q. During the process of activation, complement C1 is an initial factor, and subunit C1q plays an important role in recognition.

At present, many studies focus on the mechanism of HIRI, however, the precise mechanism is still not entirely clear. It is generally believed that HIRI is induced by the combined actions of intracellular calcium overload, generation of oxygen free radicals, Kupffer cells, cytokines and the complement system. However, the role of complement system in HIRI is rarely reported. In this study, total hepatic I/R model was established to explore the dynamic changes of complement C1q at multiple time points after total hepatic I/R, and to clarify the role of complement C1q in hepatic I/R.

1 MATERIALS AND METHODS

1.1 Experimental Animals and Grouping

Sixty healthy male SD rats (180–200 g) were provided by the Experimental Animal Center, School of Medicine, Wuhan University (China), and these rats were randomly divided into 5 groups: sham-operation group (S group, *n*=12), group of total hepatic I/R for 1 h (I/R 1 h group, *n*=12), group of total hepatic I/R for 3 h (I/R 3 h group, *n*=12), group of total hepatic I/R for 6 h (I/R 6 h

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group, n=12), and group of total hepatic I/R for 24 h (I/R 24 h group, n=12).

This study was approved by constituted ethical committee of Zhongnan Hospital, Wuhan University, China.

1.2 Establishment of Total Hepatic I/R Model

The total hepatic I/R model was established using the methods of Parasrampuria *et al*[5]. Rats were anaesthetized using 3% sodium pentobarbital at a dose of 50 mg/kg. Supine position was taken and the limbs and head were fixed. Abdominal median incision was made, and non-invasive arterial clip was used to close proper hepatic artery, portal vein, common bile duct, suprahepatic vena cava and hepatic inferior vena cava. Major hepatic blood vessels were blocked to simulate total hepatic ischemia stage. Non-invasive arterial clip was released 30 min later, and the reperfusion phase was simulated by restoring the blood supply. Next, abdominal cavity was sutured, and the rats were put back into the cages. Rats were sacrificed by taking femoral artery blood after 1 h, 3 h, 6 h, and 24 h of reperfusion, respectively. The specimens were used for testing. The operation in the S group was the same as hepatic I/R groups except that arterial clip was not given.

1.3 Specimen Collection

Rats were sacrificed by taking femoral artery blood after 1 h, 3 h, 6 h and 24 h of reperfusion, respectively. The liver tissue was washed using cold saline, and there was no residual blood. Then the tissue was wiped by clean filter paper. Tissues were prepared for fluorescence real-time quantitative PCR, Western blotting, and determination of superoxide dismutase (SOD) and malondialdehyde (MDA). The remaining specimens were immediately fixed in 4% paraformaldehyde, and further used for histopathological examination.

1.4 Liver Histopathological Examination

Liver tissue was fixed in 4% paraformaldehyde for 24 h, and further dehydrated in gradient alcohol. Tissues were vitrified using dimethylbenzene and anhydrous ethanol, and embedded using paraffin. Sections were stained using hematoxylin-eosin. Finally, the pathological changes of the liver were observed under a microscope.

1.5 Determination of SOD and MDA in Liver Tissue

Rats were sacrificed by taking femoral artery blood after 1 h, 3 h, 6 h and 24 h of reperfusion, respectively. The liver tissue was taken and frozen at –20°C in a low temperature refrigerator. The tissue was thawed in a mixture of ice and water and prepared as 10% homogenate. The SOD and MDA were determined according to the instructions of kits from Shanghai Hengyuan, China.

1.6 Real-time PCR

Totally, 100 mg of liver tissue was taken and 1 mL of Trizol reagent was added into an Eppendorf (EP) tube, and the mixture was placed on ice for precooling. Next, 250 μL of chloroform was added and the rube was inverted for 15 s. The mixture was placed for 3 min, and centrifuged at 13 000 g for 8 min at 4°C. Then the supernatant was obtained, and 0.8-fold volume of isopropanol was added and mixed. After resting for 15 min at –20°C, the mixture was centrifuged at 4°C for 10 min at 13 000 g. The supernatant liquid was discarded and 1.5 mL of 75% ethanol was added to wash the precipitate. Then the solution was centrifuged at 13 000 g for 5 min at 4°C. Finally, the tube was placed in a clean bench and blown for 3 min, and RNA was dissolved in 20 μL of RNA enzyme-free water. Furthermore, 2 μg of RNA and 1 μL of Oligo (dT) 15 were added in a PCR tube, and RNase-free deionized water complemented to totally 12 μL. Then the tube was incubated for 5 min at 70°C and quickly placed on ice for cooling. Subsequently, 4 μL of 5×buffer, 2 μL of 10 mmol/L dNTPs, 1 μL of RNA inhibitor and 1 μL of reverse transcriptase were added into the PCR tube and mixed. Then the mixture was kept at 42°C for 30 min and 80°C for 5 min, and the reverse transcriptase was inactivated. The protocol for real-time PCR was as follows: 12.5 μL of 2×qPCR Mix, 2.5 μmol/L C1q gene primers (upstream: 5'-TCACCTCA-ACTGGTTTGCTCC-3'; downstream: 5'-CAAGATT-GCCTCCATTC TCATTAC-3', 174 bp) or 2.0 μL of 2.5 μmol/L internal primer, 2.0 μL of reverse transcription products and 8.5 μL of ddH_2O. Each reverse transcription product was prepared in three tubes. The PCR process was as follows: 95°C for 1 min and 40 cycles with a final extension at 72°C for 5 min. Data were handled with ΔΔCT method[6]. A=CT (target gene, experimental samples)–CT (internal gene, experimental samples), B=CT (target gene, control samples)–CT (internal gene, control samples), K=A–B, and the expression fold was equal to 2^{-K}.

1.7 Western Blotting

Liver tissue was cut into small pieces, and cocktail, PMSF and phospho-proteasome inhibitor were added. Moreover, 10 times volume of extraction reagent was added to homogenize the tissue on ice. After 30 min of ice bath, the mixture was centrifuged at 12 000 g for 5 min, and the supernatant was collected. Protein concentration was determined using Bradford assay (GAPDH protein was used as an internal control). The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE), and the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was further blocked for 1 h at room temperature using 5% skimmed milk (prepared with 0.5% TBST). The membrane was further incubated with diluted primary-antibodies [Tris-Buffered Saline Tween-20 (TBST) dissolved in 5% skim milk] at 4°C overnight. The membrane was washed with TBST, and incubated with secondary antibodies at room temperature for 30 min. The membrane was washed again with TBST, and the target proteins were detected using an enhanced chemiluminescence (ECL) kit. Furthermore, the membrane was exposed and developed. The film was scanned and preserved. The absorbance (A) value of the target band was analyzed using Alpha software processing system. The primary-antibodies, secondary antibodies, PVDF membrane and fluorescent dye (SYBR Green) were purchased from Miao Bo Bio-Technology Co., Ltd., China.

1.8 Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) version 17.0. Data were represented as $x\pm s$. Comparison between and within groups was done using single-factor analysis of variance (ANOVA). $P<0.05$ was the level of significance.

2 RESULTS

2.1 Morphological Observation of Liver Tissue

In the S group, the normal structure of liver tissue was observed. The hepatocytes regularly arranged, and the cells presented polygonal shape and clear boundary. The nuclear was clear and located in the centre with abundant cytoplasm. In the I/R 1 h–24 h groups, pathological changes deteriorated with the prolonged I/R time. The hepatocytes arranged irregularly with faint boundary, and the swelling and split cells, infiltration of lymphocytes, neutrophils adhesion, small pieces of necrosis and apoptosis were also observed (fig. 1).

2.2 Determination of SOD and MDA in Liver Tissue

There were significant differences in SOD activity and MDA level between four I/R groups and S group (P<0.05). SOD activity was gradually decreased with prolonged I/R and MDA activity gradually increased with prolonged I/R (table 1).

2.3 Complement C1q mRNA Expression

The mRNA expression of C1q and β-actin in the S group and I/R groups is shown in fig. 2. The relative C1q expression in the S group, I/R 1 h group, I/R 3 h group, I/R 6 h group, and I/R 24 h group was 1.093±0.120, 1.903±0.056, 2.783±0.223, 1.553±0.070 and 2.313±0.305, respectively. As compared with the S group, C1q mRNA expression in all I/R groups was significantly increased (P<0.05). The C1q mRNA expression peaked in I/R 3 h group (P<0.05). As compared with other I/R groups, C1q mRNA expression was decreased in I/R 6 h group (P<0.05, fig. 2).

Table 1 Comparison of SOD activity and MDA level in the liver tissue of different groups ($\bar{x}$±s; n=12)

Groups	SOD activity (U/mg protein)	MDA level (nmol/mg protein)
S	285.96±5.92	0.906±0.140
I/R 1 h	171.10±12.80*	1.680±0.134*
I/R 3 h	219.90±14.65*	1.856±0.198*
I/R 6 h	186.03±12.30*	3.806±0.326*
I/R 24 h	120.20±16.55*	4.943±0.300*

*P<0.05 *vs*. S group

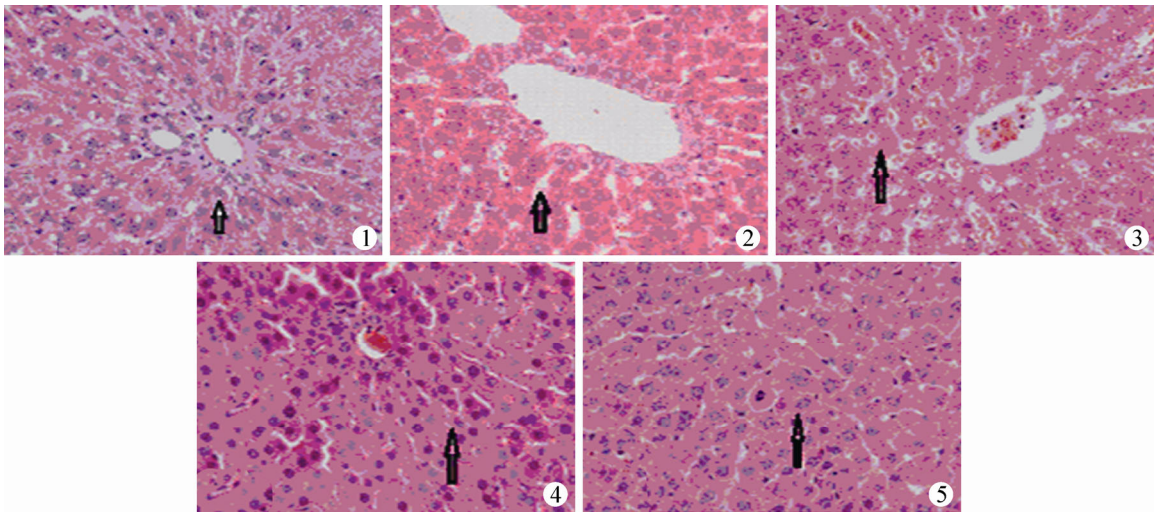

Fig. 1 Morphological observation of the liver tissue under a microscope (HE×400)
1: S group; 2: I/R 1 h group; 3: I/R 3 h group; 4: I/R 6 h group; 5: I/R 24 h group. Arrows indicated hepatocytes.

2.4 Protein Expression of Complement C1q

The complement C1q protein was detected in the S group and I/R groups, and the bands are shown in fig. 3 (internal control: GAPDH). The bands of C1q were normalized (the ratio of grey value of C1q to that of GAPDH protein). The relative protein expression of C1q in the S group, I/R 1 h group, I/R 3 h group, I/R 6 h group and I/R 24 h group was 0.345±0.060, 0.833±0.069, 1.618±0.204, 0.689±0.042 and 1.251±0.207, respectively. As compared with the S group, complement C1q protein expression in I/R groups was significantly increased (P<0.05). The protein expression of C1q peaked in I/R 3 h group (P<0.05). As compared with other I/R groups, C1q protein expression was decreased in I/R 6 h group (P<0.05, fig. 3).

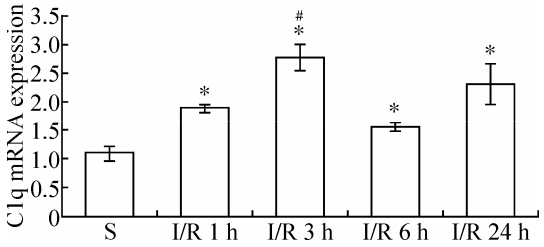

Fig. 2 mRNA expression of complement C1q
*P<0.05, #P<0.01 *vs*. S group

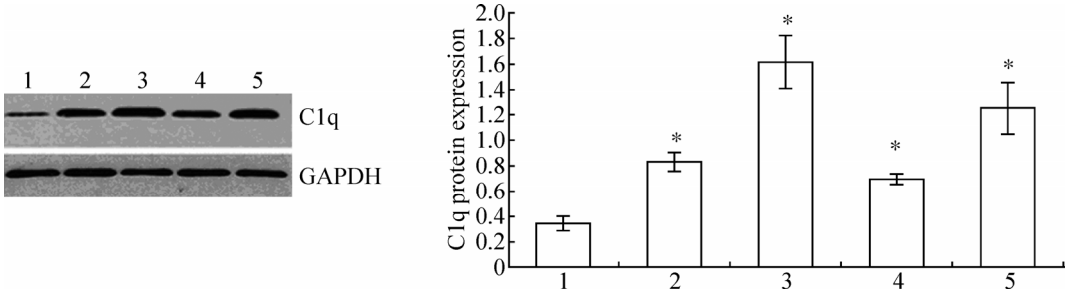

Fig. 3 C1q protein expression

1: S group; 2: I/R 1 h group; 3: I/R 3 h group; 4: I/R 6 h group; 5: I/R 24 h group. $^{*}P<0.05$, $^{\#}P<0.01$ *vs*. S group

3 DISCUSSION

Complement system is a group of proteins with enzymatic activity in serum and body fluids of humans and vertebrates. The complement system is an important effector and effector amplification system in organism with a variety of biological roles. As an important component of inflammatory response, complement has received more and more concerns. The canonical pathway of complement activation is a series of cascade reactions after combination of specific antigen-antibody complexes with C1q. C1r, C1s, C4, C2 and C3 are successively activated, C5 and C3 invertases are generated and amplified effect formed. Then the cleaved and activated complement components are distributed to the target organs and cells that are bound with antibodies. In the canonical pathway of complement activation, complement C1 is an initial factor and subunit C1q plays an important role in recognition.

Our study showed that complement C1q mRNA expression was increased in liver tissue at different time points of total hepatic I/R, suggesting that the canonical pathway of complement activation was involved in total HIRI. The C1q gene expression was increased after 1 h of total hepatic I/R. The combination of cell membrane surface antigen and its antibody can induce the overexpression of complement C1q, thus leading to changes in the liver tissue and circulatory system. With prolonged I/R time, complement C1q expression peaked at 3 h of total hepatic I/R.

In the S group, the normal structure of liver tissue was observed. The hepatocytes regularly arranged, and they presented polygonal shape and clear boundary. The nuclear was clear and located in the central region with abundant cytoplasm. In I/R 1 h–24 h groups, the pathological changes deteriorated with prolonged I/R time. The hepatocytes arranged irregularly with faint boundary, cell swelling and split, infiltration of lymphocytes, neutrophils adhesion, small pieces of necrosis and apoptosis were observed. The results also showed that SOD activity in the liver tissue was gradually decreased with prolonged I/R time, suggesting that ATP activity was decreased and metabolism was increased during liver ischemia and hypoxia. SOD was inactivated and finally exhausted.

I/R occurs for the interactions of various factors. Many studies found that the complement system plays a principal role in I/R injury[7]. Studies have shown that I/R injury in different tissues and organs depends on different pathways of complement activation. Lee *et al* reported that three pathways of the complement system were activated at different levels after intestinal I/R injury[8]. After cerebral I/R, three pathways of the complement system were activated at different levels, leading to neuronal apoptosis and vascular injury[9]. After intestinal I/R, the complement system is overly activated, resulting in production of C5a, C3a, and C5b-9, which directly or indirectly mediates the roles of inflammatory mediators. And finally the tissue damage is aggravated[10]. The findings of HIRI complement activation were consistent with other studies of I/R[7]. The inhibition of complement activation can reduce I/R injury in the liver and other organs. New strategies will be developed to prevent I/R injury in liver surgery. Studies demonstrated that ischemic preconditioning and chemical reagents can prevent HIRI[7]. Ethanol preconditioning was proved to protect the liver from I/R injury in a rat liver I/R model, and this effect directly regulated the activation of complement system[11].

In summary, the liver tissue damage gradually deteriorated with prolonged I/R time. The complement C1q played a major role in total hepatic I/R, especially at the early stage of reperfusion. More and more attentions are paid to the role of complement in I/R injury, and anti-complement therapy has become an important therapeutic target for the treatment of complement overexpression-induced diseases during I/R injury. At present, the relationship between hepatic I/R injury and the complement system is rarely reported. And the studies were limited to animal models. Therefore, the relationship between I/R injury and complement also needs to be further studied, and it will provide better foundation for clinical treatment.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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