Original Article
Role of micro-RNA-223 in hepatic ischemia-reperfusion injury

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Abstract: Background: Hepatic ischemia and reperfusion injury involve the hepatic tissue damage that is caused by the return of the blood supply to the tissue after a period of ischemia or lack of oxygen. Recent studies have suggested that there is a particularly important relationship between micro-RNA-223 and the immune system. Aim: To investigate the early diagnostic value of micro-RNA-223 in rat hepatic ischemia and reperfusion injury. Methods: Male Wistar rats were randomly divided into the following three groups: a sham operation group (group A), a 15-minute ischemia time group (group B), and a 30-minuteischemia time group (group C). Relative to the time of the establishment of hepatic ischemia-reperfusion injury, the serum levels of alanine aminotransferase and aspartate transaminase and the level of micro-RNA-223 in the liver tissue were determined after 15 min, 1 h, 3 h, 6 h, 1 d, and 3 d of reperfusion time. Results: In groups B and C, the micro-RNA-223 levels at each point were more obviously increased than the serum alanine aminotransferase and aspartate transaminase levels ($P<0.05$). Conclusion: micro-RNA-223 is a more sensitive indicator of hepatic ischemia and reperfusion injury than the traditional indices of liver function (i.e., alanine aminotransferase, aspartate transaminase).

Keywords: Liver, Ischemia reperfusion and injury, micro-RNA-223

Introduction

Hepatic ischemia and reperfusion injury (HIRI) involve the hepatic tissue damage that is caused by the return of the blood supply to the tissue after a period of ischemia or lack of oxygen and can be divided into hot ischemia-reperfusion injury and cold ischemia-reperfusion injury [1]. Hot ischemia-reperfusion injury typically occurs with digestive tract hemorrhages, uncontrolled hemorrhagic shock, liver resection surgery, etc. However, cold ischemia-reperfusion injury can be observed during liver transplantation surgeries. HIRI is a significant cause of hepatic failure after hepatectomy and hepatic insufficiency after liver transplantation and has become a major barrier to favorable surgery prognoses. Clinically, HIRI is a complex pathophysiological process that involves many factors and multiple pathways; e.g., the damage that results from oxygen free radicals, calcium overload, the activation of Kupffer cells, and the accumulation of the neutrophils, cytokines and nuclear transcription factor (NF-κB) [2].

Recent studies have suggested that there is a particularly important relationship between micro-RNA-223 (miR-223) and the immune system and that this relationship is especially important in the processes of activating neutrophils and the secretion of inflammatory cytokines [3]. Thus, our studies aimed to investigate the expression of miR-223 in the hepatic tissues of HIRI mice and to clarify the correlation between miR-223 and HIRI.

Materials and methods

Materials

All experiments were conducted using mature healthy male Wistar rats of approximately 6-7 weeks old that weighed 230-250 g and were

Table 1. The levels of alanine aminotransferase in three groups (U/L, $\bar{x}\pm s$)

Group	15 min	1 h	3 h	6 h	1 d	3 d
Sham	51.4±6.1	55.4±5.1	52.7±4.7	53.6±3.1	50.4±6.7	56.2±4.1
IR-15 min	56.4±13.4	200.9±35.8[a]	488.2±119.6[a]	904.1±282.1[a]	241.6±48.3[a]	134.4±6.4[a]
IR-30 min	320.0±149.6[b]	723.6±129.8[b]	1159.2±246.2[b]	1699.4±234.4[b]	650.1±46.4[b]	205.1±28.5[b]

Compare with Group Sham, [a]P<0.05; compare with Group IR-15 min, [b]P<0.05.

Table 2. The levels of aspartate transaminase in three groups (U/L, $\bar{x}\pm s$)

Group	15 min	1 h	3 h	6 h	1 d	3 d
Sham	140.5±28.8	150.4±19.7	135.6±35.1	137.3±30.5	120.5±25.1	146.1±30.5
IR-15 min	145.9±38.5	419.7±64.4[a]	626.2±220.7[a]	965.4±237.3[a]	342.4±112.2[a]	190.6±16.2[a]
IR-30 min	381.0±130.4[b]	642.9±180.1[b]	882.1±318.4[b]	1291.8±427.3[b]	471.3±24.7[b]	248.6±22.3[b]

Compare with Group Sham, [a]P<0.05; compare with Group IR-15 min, [b]P<0.05.

supplied by the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing. All animals were bred and maintained in airy, clean, and warm conditions (20-25°C).

Groups and HIRI models

Fifty rats were divided into five groups according to the different warm ischemia times (15 min, 30 min, 40 min, 45 min, and 60 min), and the overall survival rates were detected. Additionally, 108 rats were divided into the following three groups: a sham-operation group (Sham, group A), a 15-min ischemia and reperfusion group (IR-15, group B), and a 30-min ischemia and reperfusion group (IR-30, group C). Additionally, each group was divided into six subgroups according to the different reperfusion times (15 min, 1 h, 3 h, 6 h, 1 d, and 3 d), and each subgroup involved six rats. Pringle's method was used to set up the HIRI models. The hepatic pedicle was clipped with vascular clamps to close off the portal vein, the hepatic artery and the common bile duct. After the liver was in complete ischemia, the hepatic pedicles were opened, and the liver was reperfused for different amounts of time. In the sham-operation group, we only opened the rats' abdomen and exposed the porta hepatis without any clipping. Specimens were collected at different reperfusion times (15 min, 1 h, 3 h, 6 h, 1 d, and 3 d) from the different groups.

Blood tests

Blood samples of approximately 5 ml were collected from the abdominal aortas with trocars and centrifuged for 10 min at room temperature to obtain the sera for the alanine aminotransferase (ALT) and aspartate transaminase (AST) tests.

RT-PCR for miR-223

The tested samples were cut from the middle hepatic lobes and were approximately 1.0 cm × 1.0 cm × 0.5 cm. Total RNA was extracted from 50-100 mg of tested tissue using a mirVana miRNA isolation kit (p/n AM 1556, Ambion Inc.) according to the manufacturer's instructions. Purified total RNA was reverse-transcribed in a total volume of 20 μL and transcriptor reverse transcriptase (TaqMan MicroRNA Reverse Transcription Kit). The reaction mixture was first incubated for 10 min at 16°C and then for 45 min at 42°C followed by an enzyme inactivation step for 5 min at 85°C. The sequences of the miR-223 primers were designed according a PCR primer selection program (forward: 5'-TAG GGT ACC GCT GAA TTG GGT AGG-3', reverse: 5'-GTC TCG AGC CAA GAG CTT CTG TGG-3') [4]. Each cycle included 5 min at 95°C, 15 s at 95°C, 60 s at 60°C, and 40 cycles were used [5].

Statistical analyses

The data is presented as the means ± the SDs. One-way analyses of variance (P<0.05) were performed for multiple group comparisons and followed with 2-sided, unpaired Student's t tests. The Tukey's HSD test was used to compare 2 independent groups. SPSS statistical software, version 16.0 was used for all statisti-
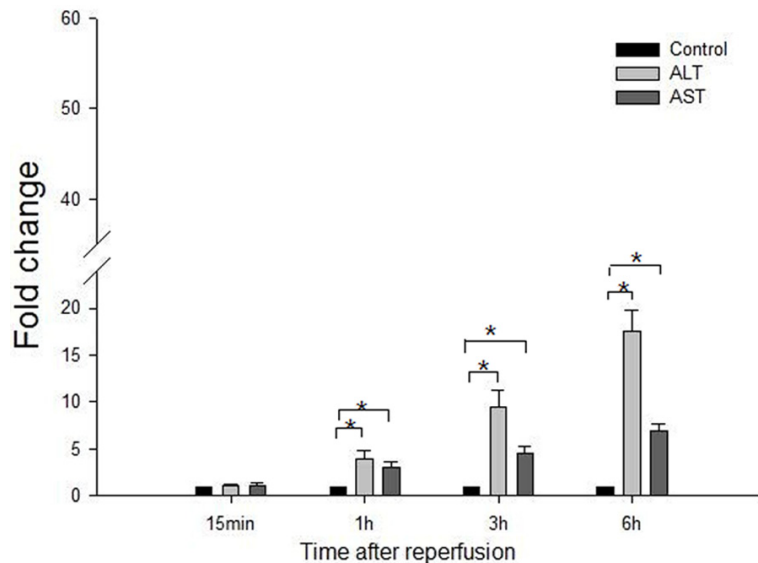
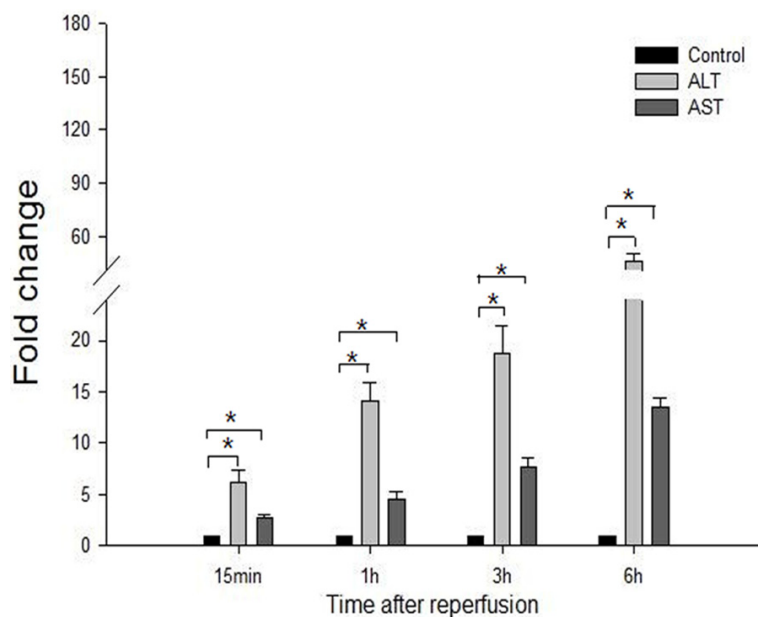**Figure 1.** The expression of liver enzyme in group B (*P<0.05).

Figure 2. The expression of liver enzyme in group C (*P<0.05).

Liver enzyme level tests

Compared to group A, the levels of ALT and AST in group B were not significantly increased after reperfusion for 15 min (P>0.05) but peaked at 6 h and were still remarkably higher at day 3 (P<0.05). However, the levels of ALT and AST in group C exhibited apparent increases at every detection time (P<0.05) compared to group B (**Tables 1** and **2**; **Figures 1** and **2**). Thus, the selected times for the tests of miR-223 were from 15 min to 6 h after reperfusion.

miR-223 expression in the liver

As shown in **Figure 3**, the expression of miR-223 in group B had increased by 2.7-fold at 15 min and by 59-fold at 6 h after reperfusion. Similarly, the expression of miR-223 in group C had increased by 20-fold at 15 min and by 150-fold at 6 h after reperfusion (**Figure 4**).

Comparison of the liver enzyme levels and miR-223 levels

In both group B and C, the expressions of miR-223 at the different test time exhibited significant increases compare to the ALT and AST levels.

Discussion

The severity of ischemia-reperfusion injury in liver transplantation directly affects the success rate of transplantation. Consequently, research into the pathogenesis of liver ischemia-reperfusion injury has become a focus in the transplant field. The mechanisms of liver ischemia-reperfusion injury are numerous; one mechanism involves the damage caused by the collection of neutrophils [6]. The roles of neutrophils in ischemia-reperfusion include the following: obstruction of microcirculation-the
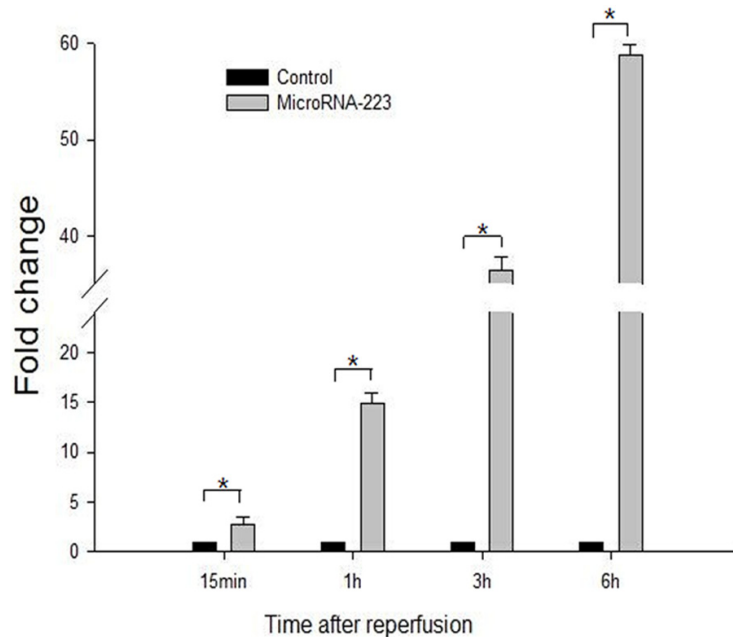
cal analyses, and P<0.05 was considered statistically significant.

Results

Determination of the warm ischemia time limit

The overall survival rates in the 15, 30, 40, 45 and 60 min groups were 100%, 100%, 40%, 20%, and 0%, respectively , and the maximum warm ischemia time that the rats could suffer was 30 min.

Figure 3. The expression of micro-RNA-223 in group B (*P<0.05).
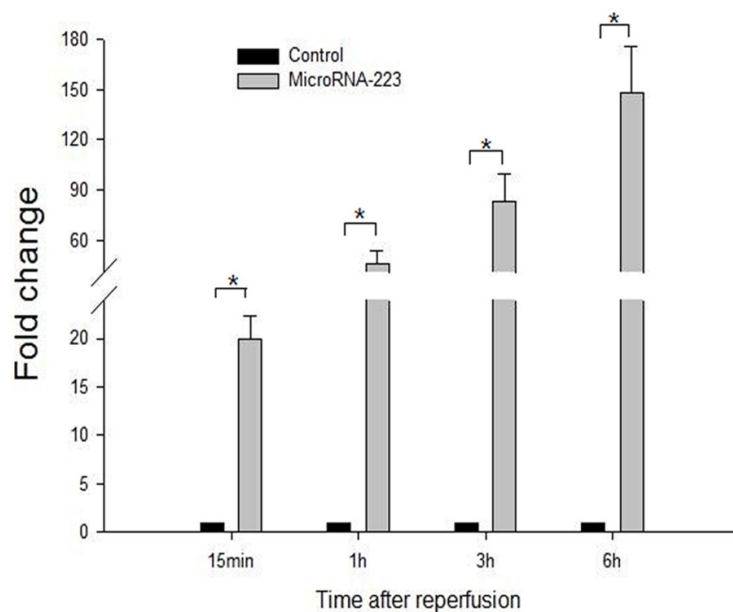
Figure 4. The expression of micro-RNA-223 in group C (*P<0.05).

non-enzymatic components are released for an extended period after leukocyte activation; and the various cytokines that are produced might injure liver cells and the extracellular matrix. Studies have found that, in the rats pretreated with neutrophil monoclonal antibodies, the necrosis of the liver and the reduction in ATP that follow ischemia-reperfusion are obviously alleviated [7].

miRNAs are a small non-coding RNA molecules (containing approximately 22 nucleotides) that are found in plants, animals, and some viruses and function in RNA silencing and the post-transcriptional regulation of gene expression. miRNAs are also known as the epigenetic regulation pathway. miRNA genes are usually transcribed by RNA polymerase and cut into 70-base hairpin structures of precursors by the Drosha enzyme. Pre-miRNA hairpins are exported out of the nucleus in a process involving the nucleocytoplasmic shuttling and Ran-GTP. Next, these structures can be cut into mature miRNAs of approximately 22 bases by the Dicer enzyme. With the guidance of the RNA-induced silencing complex (RISC), miRNAs can completely or incompletely match complementary target fragments in mRNAs. Matched of double-chain mRNA can be easily identified and degraded, which inhibits the transcription and translation of the target gene [8].

accumulation of neutrophil adhesion prevents the perfusion of the ischemia-reperfusion tissue at the microcirculatory level, which is also called the no reflow phenomenon; the release of reactive oxygen species-in the theory of free radicals, the breakage of white blood cells releases oxygen free radicals; high levels of grain components, including enzymatic and

Studies have shown that miR-223 plays an important role in the differentiation of hematopoietic cells and interacts with multiple genes and (or) transcription factors [9]. miR-223 can also affect the occurrence and development of tumors by regulating its target genes that are involved in signaling pathways. miR-223 is not only related to the formation of a wide variety of

tumors but is also a potential biomarker of some tumors [10]. However, there are still few studies in the literature that have reported on the role of miR-223 in ischemia-reperfusion injury. However, it is generally agreed that miR-223 also plays important roles in the activation and differentiation of granulocytes. One mechanism of the ischemia-reperfusion injury is the damage that is sustained due to neutrophil accumulation; therefore, it can be speculated that miR-223 might be useful as an indicator in the monitoring of liver ischemia-reperfusion injury [11].

In our studies, compared to group A, the expression of miR-223 in group B increased by 2.7-fold after reperfusion for 15 min. However, the ALT and AST levels did not exhibit significant changes. This phenomenon was more apparent in group C, which exhibited a 20-fold increase. In groups B and C, the miR-223 levels at each time point were more obviously increased than the serum ALT and AST levels ($P<0.05$). This finding suggests that miR-223 is a more sensitive indicator of hepatic ischemia and reperfusion injury than the traditional indices of liver function (i.e., ALT and AST).

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Qian YB, Liu ZG, Geng XP. Lack of protection of ischaemic preconditioning in the rat model of major hepatectomy with isehaemia reperfusion injury. Asian J Surg 2008; 31: 140-47.

[2] Jaeschke H, Woolbright BL. Current strategies to minimize hepatic ischemia-reperfusion injury by targeting reactive oxygenspecies. Transplant Rev (Orlando) 2012; 26: 103-14.

[3] Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, Li H, Wang G, Evans AR, Safe S, Wu C, Zhou B. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. Circulation 2012; 125: 2892-903.

[4] Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Müller-Tidow C, Bohlander SK, Tenen DG, Behre G. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feed-back loop in acute myeloid leukemia. Blood 2010; 115: 1768-78.

[5] Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, Seshan SV, Suthanthiran M. MicroRNA expression profiles predictive of human renal allograft status. Proc Nat Acad Sci U S A 2009; 106: 5330-35.

[6] Suzuki S, Toledo-Pereyar LH, Rodfigllez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and rcperfusian injury. Transplantation 1993; 55: 1265-72.

[7] Rentsch M, Post S, Palma P, Lang G, Menger MD, Messmer K. Anti ICAM-1 blockade reduces post sinusoidal WBC adherence following cold ischemia and reperfusion, but does not improve early graftfunction in rat liver transplantation. J Hepatol 2000; 32: 821-28.

[8] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3: 415-28.

[9] Felli N, Pedini F, Romania P, Biffoni M, Morsilli O, Castelli G, Santoro S, Chicarella S, Sorrentino A, Peschle C, Marziali G. MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis. Haematologica 2009; 94: 479-86.

[10] He X, Sai X, Chen C, Zhang Y, Xu X, Zhang D, Pan W. Host serum miR-223 is a potential new biomarker for Schistosoma japonicum infection and theresponse to chemotherapy. Parasit Vectors 2013; 6: 272-79.

[11] Sun W, Shen W, Yang S, Hu F, Li H, Zhu TH. miR-223 and miR-142 attenuate hematopoietic cell proliferation, and miR-223 positively regulates miR-142 through LMO2 isoforms and CEBP-β. Cell Res 2010; 20: 1158-69.