Circulating microRNA-122, -21 and -223 as potential markers of liver injury following warm ischaemia and reperfusion in rats

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Received July 7, 2014; Accepted March 3, 2015

DOI: 10.3892/mmr.2015.3742

Abstract. The liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are commonly used but not specific markers to quantify hepatic injury. In this *in vivo* study it was determined whether hepatic expression and serum levels of the microRNAs (miRNA) miR-122, -21 and -223 are altered and correlated with the release of liver enzymes after warm hepatic ischaemia and reperfusion (IR). Male Wistar rats were subjected to either 45 min of partial (70%) hepatic ischaemia and 240 min of reperfusion (n=7) or sham operation (n=5). Expression levels of miR-122, -21 and -223 were analysed in serum and liver tissue by quantitative polymerase chain reaction and tested for correlation with serum activities of AST, ALT and LDH. The relative expression levels of circulating miR-122 increased after IR and correlated with the serum activity of AST, ALT and LDH. Neither increased serum level of miR-21 nor elevated relative hepatic expression of miR-223 correlated with the serum activity of liver enzymes. The hepatic expression of miR-122 was unaffected by IR. The correlation between circulating miR-122 expression levels and liver enzyme activity qualifies miR-122 as a potential biomarker of warm hepatic IR injury.

Introduction

Detection and monitoring of hepatic injury after warm ischaemia reperfusion (IR) is an important but difficult task in the clinic. Measurement of serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) is the gold standard to evaluate hepatocellular damage. However, interpretation of elevated serum activities of AST, ALT and LDH may be difficult due to their occurrence in other tissues, such as skeletal or heart muscle (1).

Characteristics of an ideal biomarker for clinical use to detect hepatocellular injury involve organ specificity, fast and extensive release into the serum, fast degradation *in vivo* and high stability *ex vivo* to ensure early detection of organ injury, fast recognition of therapy success and unimpaired extended storage.

The potential role of circulating microRNAs (miRNAs) as specific biomarkers of pathological conditions was first described in several types of cancer, such as prostate cancer (2) and B-cell lymphoma (3). Elevated levels of circulating miR-21, miR-122 and miR-223 have been detected in patients with hepatocellular carcinoma (HCC) (4,5) and may aid in discriminating between patients with HCC and patients with chronic hepatitis B or cirrhosis (6). Laterza *et al* (7) showed that miR-122 is exclusively expressed in large quantities in hepatocytes. After application of different toxicants, expression of serum miR-122 specifically indicates liver cell injury with higher sensitivity compared with histology and ALT activity without any interference with muscle cell damage. A clinical study reported higher sensitivity of circulating miR-122 in comparison to ALT activity as a biomarker of toxic liver injury (8). Furthermore, the increase in miR-122 after exposure to paraquat and cholestatic liver injury correlated with the activity of ALT (9,10).

Only a few studies have analysed alterations of circulating miRNAs after hepatocellular injury by IR thus far. In a porcine model of cardiogenic shock (11) hypothermia was shown to decrease miR-122 serum expression, but not serum activity of ALT. In patients with a liver transplant, serum miR-122 correlated with the serum activity of AST and ALT (12). In a case of rejection of the transplanted liver, serum expression of miR-122 increased earlier than serum activity of AST and ALT and decreased faster after resolved rejection (12). However, the effect of exclusive warm IR on the levels of circulating miR-122 and the degree of correlation with common markers of liver injury, such as AST, ALT and LDH remains unknown.

Elevated levels of miR-21 are associated with non-alcoholic fatty liver disease and biliary tract cancer (13,14).

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Key words: circulating microRNAs, warm hepatic ischaemia and reperfusion, microRNA-122, liver enzymes

Hepatocellular expression of miR-223 correlates with elevated serum levels of AST and AST after IR in mice (15). However, whether this correlates with an increase in circulating miR-223, has not been investigated.

In blood samples, miRNAs are extremely stable and are resistant to digestion, DNase treatment, freeze/thaw cycles, boiling, low or high pH and extended storage (16,17). This could possibly be explained by the finding, that serum miRNAs are bound to high-density lipoprotein and thus, are protected against digestion (18). In addition, Turchinovic *et al* (19) stated, that a high quantity of extracellular miRNAs is bound to Argonaute-proteins, which stabilizes them for up to several months.

It was hypothesised that circulating miRNAs correlate with the liver enzymes AST, ALT and LDH and may serve as novel and improved biomarkers for liver injury after warm hepatic IR. Therefore, the present study analysed the relative expression of miR-122, miR-21 and miR-223 after hepatic IR in serum and liver tissue and tested whether the serum miRNA expression was correlated with the serum activity of liver enzymes. Furthermore, it was analysed whether increased levels of circulating miRNAs are associated with increased miRNA expression in liver tissue.

Materials and methods

Animal models. Animal maintenance and treatment were conducted in accordance with the National Institute of Health Guide for Animal Welfare. The local animal care and use committee approved this study (State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia, Germany; reference number: 8.87-50.10.34.09.017; approved by Dr. A. Blankenhorn on May 10, 2010). Male Wistar rats (animals were obtained from breedings of the Central Facility for Animal research and welfare, University Hospital, Duesseldorf, Germany; 300±20 g body weight) were housed in a controlled environment (temperature 25±1˚C, humidity 50±10%, 12 h light/12 h dark cycle) with access to food, until 16 h prior the experiment, and water *ad libitum*. After induction of anaesthesia with sodium pentobarbital (60 mg/kg intraperitoneally; Rhone-Merieux, Laupheim, Germany), the trachea was intubated and the rats were mechanically ventilated with a mixture of nitrogen (70%) and oxygen (30%; Linde gas, Höllrieglskreuth, Germany). Vena jugularis and a tail vein were cannulated for continuous administration of pentobarbital (40 mg/kg/h) and saline infusion (20 ml/kg/h; Jonosteril, Fresenius, Bad Homburg, Germany). Blood gases were analysed via the arterial line of the left carotid artery (Radiometer Medical ApS, Brønshsøj, Denmark) and ventilation was adjusted to obtain normocapnia (temperature corrected pCO_2: 40 mmHg). The adequacy of anaesthesia was assessed by arterial blood pressure and heart rate. The body temperature was monitored using a rectal temperature probe (GHM Messtechnik GmbH, Regenstauf, Germany) and was maintained between 36.5 and 37.5˚C by a heating plate (customized; connected with a heat exchanger; Dr R Lauda, Wobser GmbH & Co., KG, Lauda-Königshofen, Germany)

After midline laparotomy, the portal triad including the hepatic artery, the portal vein and the bile duct supplying the middle and left hepatic lobes was prepared, as described previously (20,21). Sham-operated animals received no further treatment (n=5). In the IR group, partial hepatic ischaemia (70%) was induced by clamping of the portal triad with an atraumatic vascular clip (Aesculap, Tuttlingen, Germany). After 45 min of ischaemia, a 240 min reperfusion period was initiated by removal of the clip (n=7).

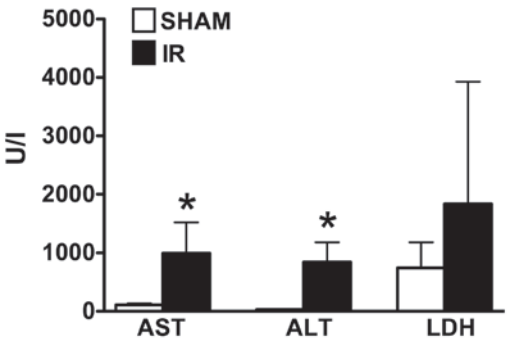

Figure 1. Activity of AST, ALT and LDH in serum of sham-operated rats (sham) or after 45 min of partial hepatic ischaemia and 240 min of reperfusion, data are presented as the mean ± standard deviation, *P<0.05 vs. Sham. IR group, n=7 and sham group, n=5. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; IR, ischaemia reperfusion.

At the end of the experiment, animals were euthanized by exsanguination after an overdose of pentobarbital. Liver tissue samples were harvested immediately and snap frozen in liquid nitrogen and stored at -80˚C for subsequent analysis. Blood samples were incubated at room temperature for 30 min and centrifuged twice at 13,000 x g for 5 min. Serum was stored at -80˚C until use.

Liver enzymes. Serum ALT, AST and LDH levels were measured by a standard automated procedure using the method of activation of pyridoxal-phosphate with subsequent photometric analysis (MODULAR analyser, Roche Diagnostics, Mannheim, Germany). Values are expressed as U/l.

Isolation of microRNAs from serum and liver tissue. Circulating miRNAs were isolated with the miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *C. elegans* miR-54 (5 μl) from a 5 fmol/μl stock were added to each sample (Applied Biosystems, Carlsbad, CA, USA). RNA from liver tissue was isolated using TRIzol® (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA purity and concentration were assessed by spectophotometry at 260 and 280 nm (NanoDrop Products, Wilmington, DE, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. Reverse transcription of the total RNA into cDNA was performed using the High Capacity RNA-to-cDNA transcription kit (Applied Biosystems), according to the manufacturer's instructions. The RT-qPCR assays for rno-miR-122 (Assay ID: 002245), rno-miR-21 (Assay ID: 000397), rno-miR-223 (Assay ID: 000526), cel-mir-54 (Assay ID: 001361) and U6 (Assay ID: 001973) were purchased from Applied Biosystems and were performed, according to the manufacturer's instructions. RT-qPCR was performed according to the manufacturer's

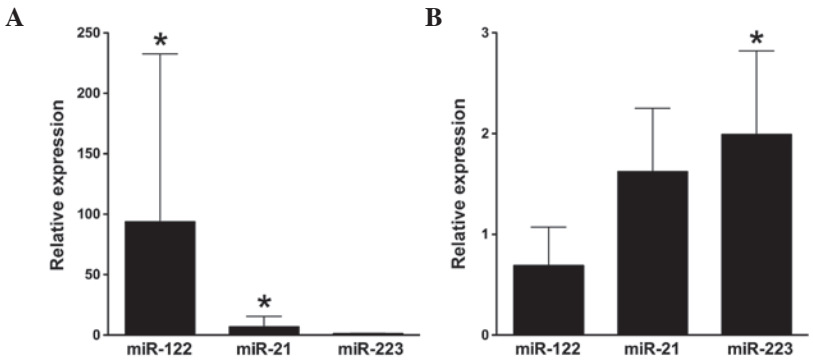

Figure 2. Relative expression of miR-122, miR-21, miR-223 in (A) serum and (B) liver tissue after 45 min of partial hepatic ischaemia and 240 min of reperfusion. The Relative Expression Software Tool was used for calculation of the fold change of miRNAs after IR (n=7). Data are presented as the mean ± standard deviation. P<0.05 vs. sham (n=5). IR, ischaemia reperfusion; miRNA, microRNA.

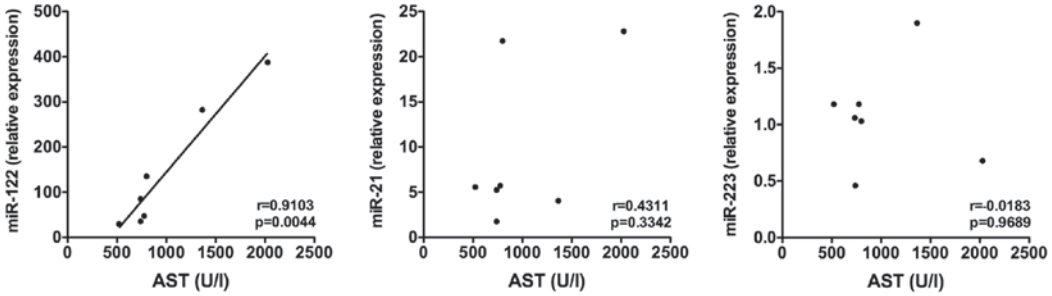

Figure 3. Correlation between relative expression of circulating miR-122, miR-21 and miR-223 and AST activity after 45 min of partial hepatic ischaemia and 240 min of reperfusion (n=7, r=coefficient of determination). AST, aspartate aminotransferase; miRNA, microRNA.

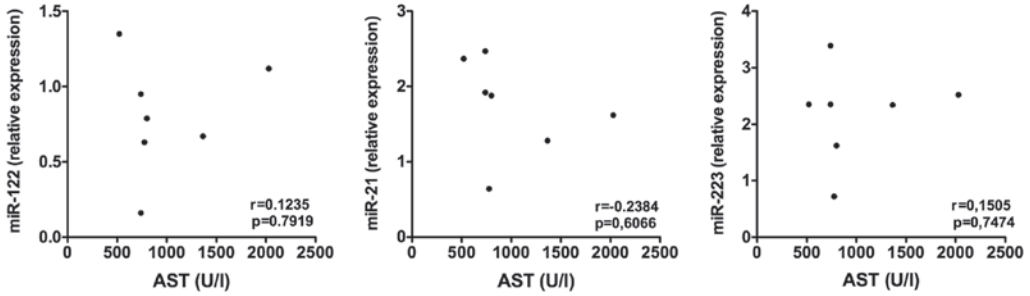

Figure 4. Correlation between relative expression of miR-122, miR-21 and miR-223 in liver tissue, and AST activity after 45 min of partial hepatic ischaemia and 240 min of reperfusion (n=7, r=coefficient of determination). AST, aspartate aminotransferase; miRNA, microRNA.

instructions on a 7300 Real-Time RT-qPCR system (Applied Biosystems). The RT-qPCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

The relative expression level of serum miRNAs was normalised to the relative expression level of miR-54 *C. elegans*, expression of liver miRNAs was normalised to U6 (Assay ID: 001973). The relative expression of miRNAs was calculated using the $2^{-\Delta\Delta Ct}$ equation (22).

Statistical analysis. Statistical calculations were performed using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA). To compare liver enzymes of sham and IR, Student's t-test was performed. Comparison of the relative expression of miR-122, -21 and -223 in serum and liver (sham vs. IR) was performed by the Relative Expression Software Tool (REST, version 2.0.13). To calculate the degree of correlation between the liver enzymes AST, ALT, LDH and the relative expression of miRNAs after IR, values were tested by linear regression analysis after logarithmic transformation (x=lnx; y=lny). P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was reviewed and approved by an independent statistician (Dr Pablo E. Verde, Coordination Centre for Clinical Studies, University Hospital Düsseldorf).

Results

Serum activity of liver enzymes after IR. To determine the severity of hepatocellular injury after IR, serum activity of AST, ALT and LDH were quantified. Serum activity of AST (IR: 995.4±523.7 vs. sham: 105.8±23. U/ l; P<0.05) and ALT (IR: 840.9±339.1 vs. sham: 30±3 U/l, P<0.05) were significantly

elevated, whereas the increase of LDH serum activity did not reach a level of significance (1836±2091 vs. 741.4±437.3 U/l; P=0.28) (Fig. 1).

Relative expression of miR-122, -21 and -223 in serum and liver. The levels of circulating miR-122 (93.65±139-fold, P=0.001) and miR-21 (6.72±8.8-fold, P=0.01) were increased after IR, while the level of miR-223 (0.99-fold±0.45, P=0.97) remained unchanged (Fig. 2A). Relative expression of miR-122 (0.69±0.38-fold, P=0.35) and miR-21 (1.62±0.63-fold, P=0.11) in liver tissue remained unchanged after IR, whereas IR induced an increase of miR-223 in liver tissue after IR compared with the sham group (1.99±0.83-fold, P=0.03) (Fig. 2B).

Correlation of relative expression of circulating and hepatic miR-122, -21 and -223 with liver enzymes AST, ALT and LDH. The serum expression of miR-122 strongly correlated with serum activity of AST, ALT and LDH (AST, r=0.9103, P=0.0044; ALT, r=0.8323, P=0.02; LDH, r=0.8772, P=0.0095). No correlation with the liver enzymes was detected for circulating miR-21 and miR-223 (Fig. 3). Relative hepatic expression levels of the investigated miRNAs did not correlate with the serum activity of AST, ALT and LDH (Fig. 4).

Discussion

In the present study, it was investigated whether circulating miR-122, -21 or -223 could serve as potential indicators of hepatocellular IR injury. Therefore serum was analysed and hepatocellular expression levels of these miRNAs after partial hepatic IR and were tested to determine whether their expression levels correlated with the activity of the established enzyme markers of hepatocellular injury AST, ALT and LDH in serum and liver tissue.

miR-122 represents a promising candidate in the context of hepatic IR, as it is highly and exclusively expressed in the liver (23). In the present study, the relative expression of circulating miR-122 increased after IR and was correlated with the serum activities of liver enzymes. This result is in line with previous studies, showing correlations between ALT and plasma miR-122 after toxic (7,24) or viral (25,26) liver injury. The potential role of circulating miR-122 as a biomarker of acute hepatic IR was first investigated by Andersson *et al* (11). In a porcine model of cardiogenic shock they showed a strong increase in circulating miR-122. The relative serum expression of miR-122 and the serum activity of ALT correlated with hemodynamic parameters in shock and no correlation between the relative expression of circulating miR-122 and the serum activity of ALT was observed. Therefore, the present results provide the first evidence for a correlation between circulating miR-122 and serum activity of ALT, AST and LDH after warm hepatic IR. However, elevated levels of circulating miR-122 following hepatic IR may be caused by an increased level of expression of miR-122 in the liver. This would in general reduce the suitability of this miRNA as a potential biomarker. Therefore, the expression of miR-122 in liver tissue was analysed and it was found that miR-122 expression was unchanged following IR. This is a novel finding, as the influence of liver IR on hepatocyte expression levels of miR-122 has not been previously determined and confirms that elevated circulating miR-122 levels following hepatic IR are not influenced by a higher hepatic expression of this miRNA. An advantage of circulating miR-122 in comparison to ALT/AST could be the faster onset of elevation. Farid *et al* showed that an elevation of circulating miR-122 occurred earlier than an elevation of AST and ALT in an acute rejection of transplanted livers (12). Additionally, the miR-122 level dropped more quickly after the initiation of a glucocorticoid therapy in these patients (27).

The present study aimed to identify other miRNAs than miR-122 that could also be indicators of hepatic IR. Recent studies have described miR-21 as a potential biomarker of non-alcoholic fatty liver disease, hepatocellular cancer (27) and chronic type B hepatitis (27). The present study shows that the relative expression of miR-21 in serum increased significantly after IR, while the expression of miR-21 was unaffected in liver tissue compared with sham. In contrast to miR-122, the elevated level of circulating miR-21 did not correlate with the activity of liver enzymes. This may be due to the lower expression level of miR-21 compared with miR-122 in liver tissue (28), which leads to a decreased release of miR-21 following hepatocellular damage.

Another promising miRNA for quantification of hepatic injury after IR is miR-223. Yu *et al* showed in a murine *in vivo* model of hepatic IR an increase in the expression level of miR-223 in liver tissue (15). Furthermore, relative hepatic expression of miR-223 correlated well with ALT and AST activity in the context of IR. However, the expression of circulating miR-223 was not analysed. In the present study these findings of elevated expression of miR-223 in the liver after IR were confirmed; however, no correlation with the serum activity of liver enzymes could be detected. In addition, the level of circulating miR-223 remained unchanged. The role of miR-223 in the context of hepatic IR is unclear. Myeloid cells show a high expression of miR-223 with granulocytes expressing the highest levels (29). Therefore, the elevated level of miR-223 in the liver following IR most likely reflects the invasion of inflammatory cells, particularly granulocytes (30).

In conclusion, it was demonstrated that circulating miR-122 levels are elevated after exclusive warm hepatic IR. Furthermore, the level of circulating miR-122 correlates with ALT, AST and LDH levels and therefore represents a promising candidate biomarker of liver injury following warm IR.

Acknowledgements

The authors would like to thank Dr Carmen Barthuber, Mrs. Yvonne Grueber and Mrs. Antje Nebert for helpful assistance with the study. They would also like to acknowledge the statistical review and approvement of Dr Pablo E. Verde, Coordination Centre for Clinical Studies, University Hospital Duesseldorf. The work was funded by a starter grant of the Research Committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf.

References

1. Ozer J, Ratner M, Shaw M, Bailey W and Schomaker S: The current state of serum biomarkers of hepatotoxicity. Toxicology 245: 194-205, 2008.

2. Mitchell PS, Parkin RK, Kroh EM, *et al*: Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105: 10513-10518, 2008.
3. Lawrie CH, Gal S, Dunlop HM, *et al*: Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141: 672-675, 2008.
4. Xu J, Wu C, Che X, *et al*: Circulating microRNAs, miR-21, miR-122 and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog 50: 136-142, 2011.
5. Tomimaru Y, Eguchi H, Nagano H, *et al*: Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol 56: 167-175, 2012.
6. Zhou J, Yu L, Gao X, *et al*: Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol 29: 4781-4788, 2011.
7. Laterza OF, Lim L, Garrett-Engele PW, *et al*: Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem 55: 1977-1983, 2009.
8. Antoine DJ, Dear JW, Lewis PS, *et al*: Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. Hepatology 58: 777-787, 2013.
9. Ding X, Ding J, Ning J, *et al*: Circulating microRNA-122 as a potential biomarker for liver injury. Mol Med Rep 5: 1428-1432, 2012.
10. Shifeng H, Danni W, Pu C, Ping Y, Ju C and Liping Z: Circulating liver-specific miR-122 as a novel potential biomarker for diagnosis of cholestatic liver injury. PLoS One 8: e73133, 2013.
11. Andersson P, Gidlöf O, Braun OO, Götberg M, van der Pals J, Olde B and Erlinge D: Plasma levels of liver-specific miR-122 is massively increased in a porcine cardiogenic shock model and attenuated by hypothermia. Shock 37: 234-238, 2012.
12. Farid WR, Pan Q, van der Meer AJ, *et al*: Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl 18: 290-297, 2012.
13. Yamada H, Suzuki K, Ichino N, *et al*: Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. Clin Chim Acta 424: 99-103, 2013.
14. Kishimoto T, Eguchi H, Nagano H, *et al*: Plasma miR-21 is a novel diagnostic biomarker for biliary tract cancer. Cancer Sci 104: 1626-1631, 2013.
15. Yu CH, Xu CF and Li YM: Association of MicroRNA-223 expression with hepatic ischemia/reperfusion injury in mice. Dig Dis Sci 54: 2362-2366, 2009.
16. Chen X, Ba Y, Ma L, *et al*: Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18: 997-1006, 2008.
17. Li Y, Jiang Z, Xu L, Yao H, Guo J and Ding X: Stability analysis of liver cancer-related microRNAs. Acta Biochim Biophys Sin (Shanghai) 43: 69-78, 2011.
18. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD and Remaley AT: MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 13: 423-433, 2011.
19. Turchinovich A, Weiz L, Langheinz A and Burwinkel B: Characterization of extracellular circulating microRNA. Nucleic Acids Res 39: 7223-7233, 2011.
20. Schmidt R, Tritschler E, Hoetzel A, *et al*: Heme oxygenase-1 induction by the clinically used anesthetic isoflurane protects rat livers from ischemia/reperfusion injury. Ann Surg 245: 931-942, 2007.
21. Braun S, Plitzko G, Bicknell L, *et al*: Pretreatment with helium does not attenuate liver injury after warm ischemia reperfusion. Shock 41: 413-419, 2014.
22. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.
23. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W and Tuschl T: Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735-739, 2002.
24. Wang K, Zhang S, Marzolf B, *et al*: Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci USA 106: 4402-4407, 2009.
25. Waidmann O, Bihrer V, Pleli T, *et al*: Serum microRNA-122 levels in different groups of patients with chronic hepatitis B virus infection. J Viral Hepat 19: e58-e65, 2012.
26. Cermelli S, Ruggieri A, Marrero JA, Ioannou GN and Beretta L: Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One 6: e23937, 2011.
27. Xu J, Wu C, Che X, *et al*: Circulating MicroRNAs, miR-21, miR-122 and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog 50: 136-142, 2011.
28. Landgraf P, Rusu M, Sheridan R, *et al*: A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129: 1401-1414, 2007.
29. Ramkissoon SH, Mainwaring LA, Ogasawara Y, *et al*: Hematopoietic-specific microRNA expression in human cells. Leuk Res 30: 643-647, 2006.
30. Honda M, Takeichi T, Asonuma K, Tanaka K, Kusunoki M and Inomata Y: Intravital imaging of neutrophil recruitment in hepatic ischemia-reperfusion injury in mice: Transplantation 95: 551-558, 2013.