



Is the early or delayed remote ischemic preconditioning the more effective from a microcirculatory and histological point of view in a rat model of partial liver ischemia-reperfusion?¹

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Abstract

Purpose: To compare early- and late-effect remote ischemic preconditioning (RIPC) by analysing the microcirculatory, hemodynamic and histological changes in partial liver ischemia-reperfusion of rats.

Methods: 60-minute partial liver ischemia followed by 120-minute reperfusion was performed without (Control group, n=7) or with preconditioning. In RIPC groups a tourniquet was applied around the left thigh using 3 cycles of 10-minute ischemia/10-minute reperfusion, one (RIPC-1, n=7) or twenty-four hours (RIPC-24, n=7) before I/R. Hemodynamic and microcirculatory measurements were performed before and after ischemia and in 30th, 60th and 120th minute of reperfusion and histological examination at the end of reperfusion.

Results: Blood pressure decreased in all groups followed by biphasic changes in Control group. In RIPC groups R120 values returned almost to normal. Heart rate increased in Control and RIPC-1 groups at R120, while RIPC-24 did not show significant changes. Microcirculation of non-ischemic liver stayed constant in Control and showed significant changes in RIPC-24 group, while in ischemic liver elevated by R120 in all groups. RIPC didn't reduce histological alterations.

Conclusion: Considering the survival and the results, both remote ischemic preconditioning protocols had beneficial effect in hepatic ischemia-reperfusion, however the histopathological findings were controversial.

Key words: Ischemia. Reperfusion. Ischemic Preconditioning. Microcirculation. Histology. Liver. Rats.

■ Introduction

During liver resections or liver transplantation intraoperative blood loss is a major problem. A common strategy to avoid it is clamping of the portal triad (Pringle manoeuvre) which may lead to ischemia-reperfusion injury of the liver^{1,2}. It is well known that ischemia in itself may cause serious damage, however, the restoration of blood flow exacerbates these changes further. The phenomenon is called ischemia-reperfusion injury (IRI) and may contribute to increased postoperative mortality and morbidity following liver surgery^{3,4}.

Ischemia-reperfusion injury is a complex, multifactorial pathophysiologic process. It causes direct cellular damage and delayed dysfunction through the activation of inflammatory pathways⁵ but the oxidative stress also contributes to organ dysfunction by several mechanisms.

Previous studies have shown that the hepatic microcirculation is a major target of hepatic IRI⁶. Therefore monitoring the microcirculatory conditions is inevitable in the detection and prediction of organ failure caused by IRI.

Many pharmacological agents and surgical interventions were developed and tested to decrease the ischemia-reperfusion injury^{7,8}. Most of these studies have been performed in experimental animals only. Until now only two surgical methods such as intermittent clamping (IC) and ischemic preconditioning (IPC) have been introduced into clinical practice⁹⁻¹¹.

IC means intermittent vascular inflow occlusion with short periods of reperfusion in between. The concept of IPC was first introduced by Murry *et al.*¹². The procedure consists of brief periods of ischemia-reperfusion prior to sustained ischemia. IPC is considered to be an adaptive pathophysiologic phenomenon and has been shown to reduce

hepatic ischemia-reperfusion injury in both experimental and clinical studies⁹. IPC may be applied directly or remotely. While there are contradictory data about the favourable application of IC, IPC is a technique that seems to be safe and feasible in the clinical practice too⁹. The disadvantage of the method is the direct stress to the organ and the mechanical trauma to the vessels¹³.

The concept was developed further by demonstrating that brief periods of ischemia and reperfusion applied on a remote organ or tissue can be associated with decreased damage of the target organ. The method known as remote ischemic preconditioning (RIPC) was originally developed by Przyklenk *et al.*¹⁴. Numerous studies have described the beneficial, protective effect of remote ischemic preconditioning regarding several organs, including the liver¹⁵⁻¹⁷. It was successfully applied not only in experimental studies but in clinical conditions too, however, it is still not clarified what the optimal duration and timing (number of cycles, early or late) of the process is.

The current study was designed to compare the effect of early and late remote ischemic preconditioning protocol on microcirculatory and hemodynamic changes caused by partial liver ischemia-reperfusion in the rat. Histological examination of the liver may provide further information for better understanding the pathophysiological changes and how RIPC influences the severity of the damage caused by ischemia-reperfusion.

■ Methods

Experimental animals, operative techniques and sampling protocol

All procedures were approved and registered by the University of Debrecen Committee of Animal Welfare (permission

Nr.: 25/2016. UDCAW) in accordance with national and EU regulations [Hungarian Animal Protection Act (Law XVIII/1998) and Directive 2010/63/EU].

The experiment was done using twenty-one healthy, mature Crl: WI outbred rats (Toxi-Coop Ltd., Hungary; body-weight: 384.9±68 g). The age of the animal can be a crucial factor in surgical interventions. Older rats are more sensitive for fat accumulations¹⁸, therefore young animals from the same age (14-16 weeks old) were selected. The rats were randomly allocated to three experimental groups, each group contained 5 male and 2 female rats. As analgesia, Flunixin (2.5 mg/kg s.c.) was administered one day before the operation to all animals.

The preconditioning procedures and the surgical interventions were performed in general anesthesia using sodium-thiopental (Thiopental: 60 mg/kg intraperitoneally, supplemented when necessary). The animals were placed in supine position on a heating pad to maintain body-temperature.

In both remote ischemic preconditioning groups a tourniquet was applied in three cycles by a metal based suture material around the left thigh above the inguinal ligament to occlude the complete hind-limb blood supply. The RIPC protocol included 3x10 minutes ischemia interspersed with 10-minute reperfusion periods, 1 hour (RIPC-1 group: n=7) or 24 hours (RIPC-24 group: n=7) before the sustained ischemia.

In each group the left inguinal region and the abdomen of the experimental animals were completely shaved and disinfected with Betadine. In the left inguinal region a 1 cm long horizontal incision was performed and after smooth preparation the femoral artery was cannulated. Laparotomy was performed via a midline incision, the intestine was placed gently on the left side of the animal and was

covered by a plastic bag to avoid dehydration.

After exposing the organ partial liver ischemia was achieved by using atraumatic microvascular clip on the portal vein and proper hepatic artery above the caudate process and right anterior lobe for 60 minutes to provide 70% partial liver ischemia^{19,20}. Using this model circulation of the intact lobes is preserved, while the above mentioned lobes are excluded from the liver's blood-flow. After removing the microsurgical clip 120 minutes reperfusion period was observed. During the 60-minute ischemic and 120-minute reperfusion period the anterior abdominal wound was covered with two layers, as a first layer a plastic bag was used to minimize evaporative loss and as a second layer a gauze sponge was placed on the abdomen.

Respiratory rate, rectal temperature, liver surface temperature, hemodynamic and microcirculatory measurements were performed before the operation, at the 60th minute of the ischemia (I-60) and at the 30th, 60th and 120th minute of the reperfusion (R-30, R-60, R-120).

At the end of the follow-up period and the tissue harvest, the animals were euthanized by exsanguination under anesthesia.

Hemodynamic measurements

For indirect monitoring of blood pressure (systolic, diastolic, MAP) and heart rate Apollo non-invasive Blood Pressure Amplifier (IITC Inc., CA, US) was applied. This measurement technique uses a cuff in which the tail of the experimental animal is inserted.

Respiratory rate

For following the general state of the experimental animal during the operation and the ischemia-reperfusion period the respiratory rate was observed and documented.

Rectal temperature

Rectal temperature measurement was performed by conventional digital thermometer as a common accepted and easy method for monitoring the body temperature in rodents.

Liver surface temperature

The liver surface temperature was measured at two different points, on the left lateral (ischemic-liver) and right lateral lobe (non-ischemic-liver), using an infrared thermometer.

Microcirculatory measurements

Laser Doppler flowmetry is a non-invasive, widely used technique to monitor the fluctuation in the microcirculation and the viability of the tissue. The method is based on the Doppler frequency shift phenomenon. The emitted, coherent light show changes in wavelength when it meets moving particles, but it stays unchanged when it enters static tissue elements. The laser Doppler flowmeter emits the laser beam which is reflected from the surface of the moving red blood cells, then the shift in the light is plotted on the computer's screen²¹.

Laser Doppler (LD) flowmetry (LD-01 Laser Doppler Tissue Flowmeter Experimentia Ltd., Hungary) was used for monitoring the changes in the microcirculation. By using a standard pencil probe (MNP100XP, Oxford Optronix Ltd., UK) the microcirculation of the ischemic (Left lateral lobe) and non-ischemic (Right lateral lobe) part of the liver, the kidney and the intestine were detected. During the measurement it is necessary to stabilize the signal to determine the BFU (blood flux unit) of the examined 20-second period. The findings of the microcirculatory pattern were registered and analysed²².

Histopathological examination

At the end of the observed I/R period samples were collected from the identical signs of the left lateral lobe of the liver, kidney, small-intestine and lung for histopathological examination. The excised tissue samples were fixed in 10% formaldehyde then were sent to the Department of Pathology, where they were embedded in paraffin and 4 mm serial sections were performed. Hematoxylin and eosin (H & E) and PAS staining were used to detect and analyse the morphological changes caused by the I/R injury and the intergroup differences after the applied preconditioning protocol. For evaluation of the severity of I/R caused damage Suzuki's score was used²³. Three different characteristic histological signs (sinusoidal congestion, necrosis, vacuolization) of I/R injury were individually graded on a scale from 0 to 4 by an experienced pathologist.

Statistical analysis

All data are expressed as mean \pm standard deviation (S.D.). For the simple comparison of intergroup changes Student's *t*-test or Mann-Whitney rank sum test, while changes during the examined period at the observed timepoints were determined by one-way ANOVA methods (Bonferroni's or Dunn's test), depending on the data distribution. Significance was assigned a *p* value of <0.05.

■ **Results**

Hemodynamic measurements

In the Control group the systolic blood pressure showed a continuous decrease at the end of the ischemic period and at the 30th minute of reperfusion, then the value elevated approximately to the same level of the base value. By R120 it dramatically decreased again and this alteration was significant versus the

base value of the Control group ($p=0.022$ vs. Base). The systolic blood pressure level of both preconditioning group was lower compare to the Control group at the beginning of the operation, then moderately decreased, however by the 120th minute of the reperfusion the values exceeded the systolic blood pressure of the Control group (Figure 1).

The alterations of the heart rate were fluctuated moderately in the Control group during the observed period, and the level

diminished significantly at the 60th minute of the reperfusion compare to the base values ($p=0.045$ vs. Base). In the RIPC-1 group the heart rate decreased at the 30th and 60th minute of the reperfusion and by the end of the reperfusion elevated again. The heart rate counts of the RIPC-24 group were approximately equal during the experimental protocol (Figure 1).

In spite of the observed alterations there were no statistically significant intergroup differences.

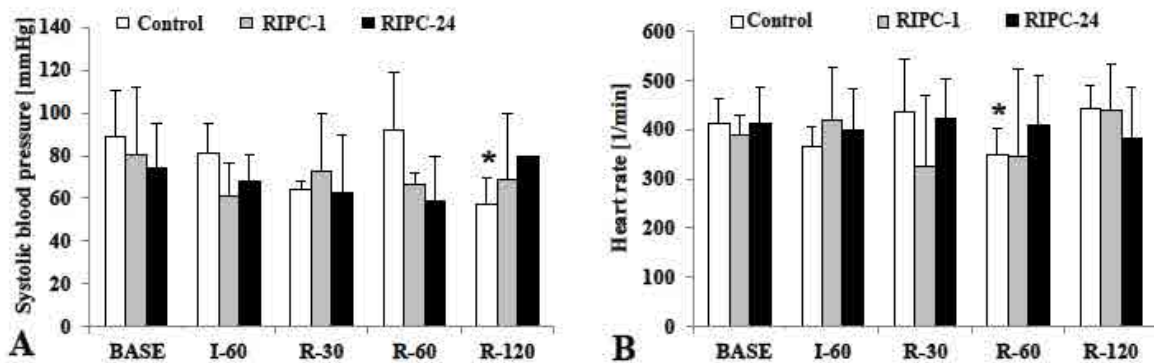


Figure 1 - Changes of systolic blood pressure [mmHg] and heart rate [1/min] in the Control, the Remote ischemic preconditioning 1 hour (RIPC-1), and the Remote ischemic preconditioning 24 hour (RIPC-24) groups. Systolic blood pressure (A), Heart rate (B). Base = before the operation; I-60 = the end of the ischemia; R30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means \pm S.D.; * $p < 0.05$ vs. Base.

Respiratory rate

There was no significant disparity in the changes of the respiratory rate, however a moderate elevation was observed in the Control and in the RIPC-24 during the reperfusion period of the process (Figure 2).

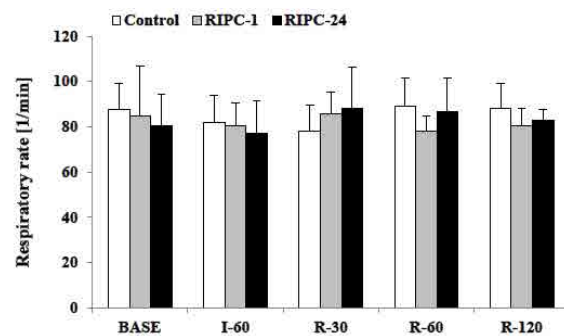


Figure 2 - Changes of respiratory rate [1/min] in the Control, the Remote ischemic preconditioning 1 hour (RIPC-1), and the Remote ischemic preconditioning 24 hour (RIPC-24) groups. Base = before the operation; I-60 = the end of the ischemia; R-30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means \pm S.D.

Rectal and liver surface temperature

The measured rectal temperature values remained constant in all groups in the different time-points of the process (Figure 3).

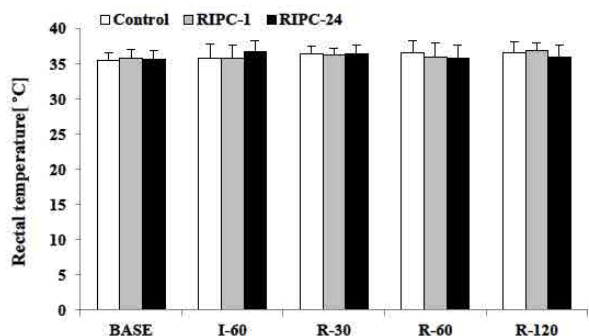


Figure 3 - Changes of rectal temperature [°C] in the Control, the Remote ischemic preconditioning 1 hour (RIPC-1), and the Remote ischemic preconditioning 24 hour (RIPC-24) groups. Base = before the operation; I-60 = the end of the ischemia; R30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means ± S.D.

Analysing the surface temperatures of the ischemic and non-ischemic part of the liver it can be established, that the temperature of the non-ischemic lobe elevated in the Control and in the RIPC-1 group at the end of the reperfusion (Control vs. Base $p=0.049$, RIPC-1 vs. Base $p=0.012$). In the RIPC-24 group the values increased significantly at the 60th minute of the reperfusion (RIPC-24 vs. Base $p=0.002$). Comparing the experimental groups the same range of alterations was found, significant intergroup difference was observed between the RIPC-24 and the Control at the 60th minute of the reperfusion ($p=0.032$ vs. Control). The temperature data of the ischemic liver were similar to the non-ischemic liver and the same tendency of changes were observed, although the difference was significant in the Control group at the 30th minute of the reperfusion ($p=0.015$ vs. Non-ischemic liver) (Figure 4).

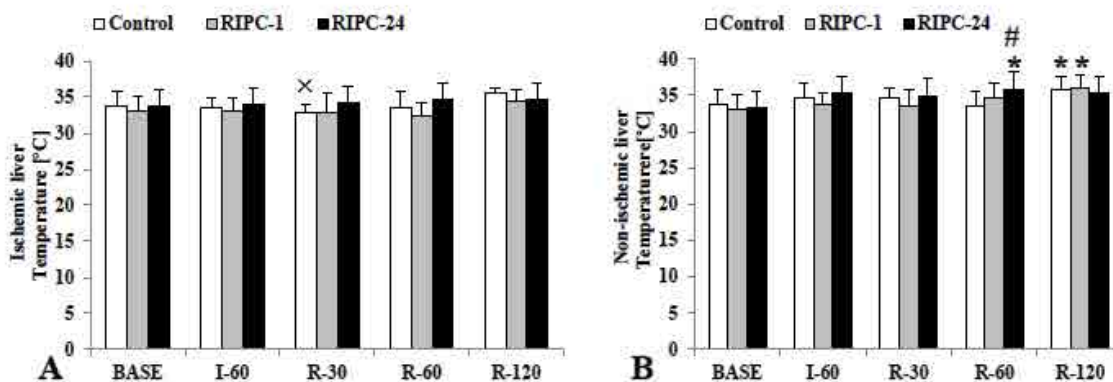


Figure 4 - Changes of liver surface temperature [°C] in the Control, the Remote ischemic preconditioning 1 hour (RIPC-1), and the Remote ischemic preconditioning 24 hour (RIPC-24) groups. Non-ischemic liver (A), Ischemic liver (B). Base = before the operation; I-60 = the end of the ischemia; R30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means ± S.D.; * $p<0.05$ vs. Base; # $p<0.05$ vs. Control; × $p<0.05$ vs. Non-ischemic liver.

Microcirculatory measurements

With regard to the non-ischemic lobe of the liver the BFU values of the Control group did not show remarkable changes in the observed follow-up period. In the RIPC-1 group the values elevated moderately by the end of

the ischemic period, but during the reperfusion decreased again. In the RIPC-24 group the BFU parameters continuously decreased till the 30th minute of the reperfusion, where the difference was significant compared to the base and the Control group ($p=0.021$ vs. Base; $p=0.036$ vs. Control), then increased again.

Examining the BFU values of the ischemic part of the liver an identical pattern of alterations was seen before the operation and at the end of the ischemia. At R-30 the BFU level of RIPC-24 group was significantly decreased ($p=0.021$ vs. Base; $p=0.036$ vs. Control). By the end of the examined reperfusion period the values were moderately elevated in all groups.

The BFU values of the non-ischemic liver versus the ischemic lobe were significantly lower in the Control group at R120 ($p=0.035$ vs. ischemic liver) In the RIPC-1 group the BFU values of the ischemic lobe were lower at R-60 compared to the non-ischemic part of the liver

($p=0.043$ vs. Non-ischemic liver).

The BFU parameters of the small intestine were increased at I-60 in the Control and in the RIPC-24 groups, then decreased during the reperfusion. At R-60 the difference was significant between the Control and RIPC-1 groups ($p=0.038$ vs. Control).

With regard to the microcirculation of the right kidney consistently higher values were registered in the RIPC-1 group compared to the RIPC-24 group. In the RIPC-24 group the changes were significant versus Control at I60 and R60 ($p=0.001$ and $p=0.010$ vs. Control) (Figure 5).

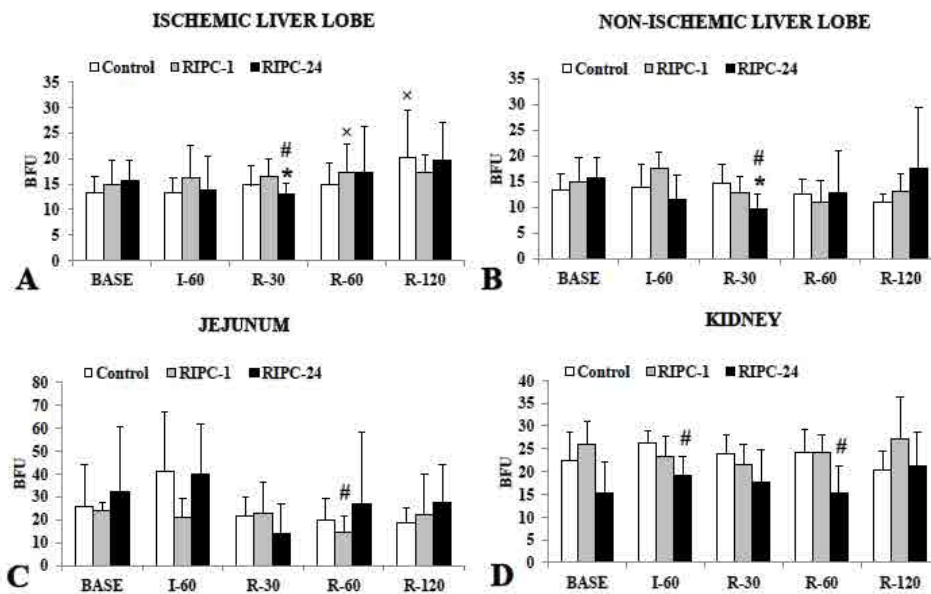


Figure 5 - Changes of the BFU values in the Control, the Remote ischemic preconditioning 1 hour (RIPC-1), and the Remote ischemic preconditioning 24 hour (RIPC-24) groups. Non-ischemic liver (A), Ischemic liver (B), Intestine (C), Kidney (D). Base = before the operation; I-60 = the end of the ischemia; R30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means \pm S.D.; * $p<0.05$ vs. Base; # $p<0.05$ vs. Control; \times $p<0.05$ vs. Non-ischemic liver.

Histopathological examinations

In our current study the following histological alterations were observed after ischemia-reperfusion injury (Figure 6): signs of sinusoidal congestion, pericentral desorganisation, necrosis in the pericentral region, hepatocyte dilatation, signs of

inflammation in the central zone the cytoplasm is more eosinophil and homogenous. Quantifying the observed histological alterations of the ischemic lobe of the liver moderate sinusoidal congestion, necrosis and in 50% of the examined samples mild vacuolization could be detected in the sections of the Control group. Regarding the results of

the RIPC-1 group it can be determined that the deterioration of the tissue damage was more serious compared to the Control group, as higher amount of hepatocyte necrosis close to the sinusoids, congestion and also inflammatory cells were described. Similar

histopathological changes were found in the RIPC-24 group, but the vacuolization was more severe and even the total score was higher compared to the Control and the RIPC-1 group (Control: 4.125 ± 1.96 ; RIPC-1: 5.83 ± 1.6 ; RIPC-24: 6.13 ± 2.12).

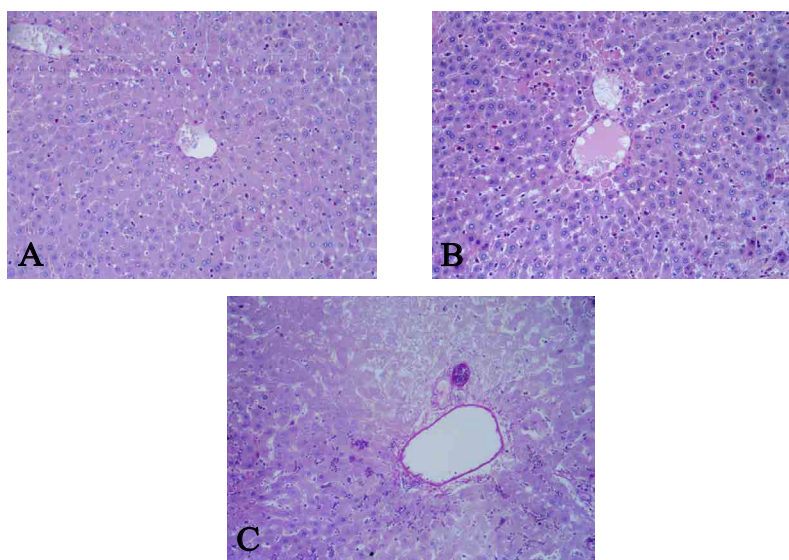


Figure 6 - Histology of the ischemic lobe of the liver (H & E staining; original magnification: 20x). Samples from the Control (A), Remote ischemic preconditioning 1 hour before I/R (RIPC-1) (B), and Remote ischemic preconditioning 24 hour before I/R (RIPC-24) (C) groups.

■ Discussion

The purpose of our present study was to compare the effect of early and late remote ischemic preconditioning protocols on hepatic ischemia-reperfusion injury by evaluating the hemodynamic, microcirculatory and histological changes.

Ischemia-reperfusion of the liver inevitably occurs during liver surgery by applying the Pringle manoeuvre, resulting in insufficiency of the liver remnant, associated with increased morbidity and mortality. Several surgical strategies have been developed and examined to reduce I/R injury including remote ischemic preconditioning. Lai *et al.*²⁴ proved the effectivity of RIPC in rat liver model in 2006. Numerous studies have been designed to reveal the exact mechanism, pathways

and mediators of preconditioning, but a clear understanding is still lacking. There is also no consensus on the most effective timing of IPC protocols. Total duration, number of cycles, tissue volume, duration of the ischemic and reperfusion periods may all influence the outcome²⁵.

The optimal interval between the preconditioning stimulus and the onset of sustained ischemia is also not known. In respect of coronary vessels it was concluded that if this interval exceeds 2 hours, the beneficial effects begin to wane. If extended to 24-72 hours, a 'second window of protection' emerges²⁶. Another study found that in cardiac surgery the effect of RIPC was better if used 24 h before the ischemic insult²⁷.

In our research RIPC was performed with the most commonly used protocol of

3 cycles of 10-minute ischemia/10-minute reperfusion performed 1 or 24 hours before introducing partial hepatic ischemia²⁸.

Concerning the hemodynamic parameters the alterations in the systolic blood pressure and the heart rate were steadier in RIPC groups compared to the control group. The heart rate was stable in RIPC-24 group, while it was rather fluctuating in RIPC-1 and C groups throughout the time course of observation. These findings partly differ from that of Tapuria *et al.*²⁹. In their research a transient drop in BP with recovery to baseline by R30/R60 in both I/R and RIPC groups was observed.

Neither the respiratory rate nor the rectal temperature showed significant changes during the observation period. The liver surface temperature values followed a similar pattern in all groups showing a moderate elevation that reached significance vs. base in RIPC-24 at R60 and in RIPC-1 and C groups by R120.

To monitor the microcirculation we used laser Doppler flowmetry. It is a widely used, non-invasive method, although having some drawbacks. The most important one is that several factors may influence the result including temperature, instability of the probe, organ movements, evaporation of the tissue, local blood pressure and oxygenation³⁰.

We found that by the end of the observed period the BFU values reached or exceeded the base values in both RIPC groups. The 1-hour preconditioning protocol could be more effective in preserving the microcirculation of the liver as in most of the examined time points higher BFU levels were registered in RIPC-1 than in RIPC-24 group. However, by the end of the I/R period the highest BFU values were observed in the RIPC-24 group.

Several studies demonstrated that RIPC protects the liver from IRI by modulation of the hepatic microcirculation. This protective effect is thought to be the result of increased RBC velocity, sinusoidal perfusion and

decreased neutrophil adhesion and cell death, though the underlying mechanism is still not understood^{28,31}.

Tapuria *et al.*²⁹ found using intravital videofluorescence microscopy that RIPC had a positive effect on microcirculation as it resulted in an increased velocity of RBC flow, sinusoidal flow and sinusoidal perfusion compared to I/R group at all time points. The significant increase in sinusoidal flow was due to increase in diameter as had been demonstrated by the author in earlier studies as well^{31,32}.

It is known that micro-rheological parameters such as red blood cell deformability and aggregation play important role in influencing microcirculation²². In our former manuscript using the same model we reported the effects of RIPC on micro-rheological parameters. It was found that erythrocyte deformability was improved in the RIPC-1 group that supports the present findings.

Histologically endothelial cell disruption, cellular swelling, vacuolization, neutrophil infiltration and hepatocellular necrosis characterize the hepatic I/R injury.¹

Freitas *et al.*³³ examined various ischemia-reperfusion periods using Pringle manoeuvre (10-, 20- and 30-minute ischemia followed by 15-, 30-, 60- and 120-minute reperfusion) in rats and found that 'the longer the duration of ischemia and reperfusion, the greater are the morphologic changes'. Accordingly, 60-minute ischemia followed by 12-minute reperfusion employed in our study was expected to cause significant histological alterations.

Unexpectedly, these signs were more pronounced in both RIPC groups compared to the Control group. These results are against the findings of others, where the pathological changes in the RIPC groups were milder than those in the I/R group^{29,34}. The result of the Suzuki's score system were consistent to these observations. A possible reading of the controversial histological findings is the

influence of acute phase reactions that is more pronounced in the RIPC-24 group due to the longer time period. The findings of Vega *et al.*³⁵ provide further explanation. They have shown that xanthin-oxidase released from the ischemic hind limb during reperfusion is taken up by the liver where it mediates Kupffer-cell and polymorphonuclear leukocyte activation and thus contributes to the development of multiple organ failure.

A limitation of our study is that only healthy, young animals were exposed to ischemia-reperfusion. Based on the literature it is known that old age, steatotic or cirrhotic livers and certain co-morbidities are aggravating factors in ischemia-reperfusion injury⁹.

Limani *et al.*³⁶ used a mice model for testing whether RIPC is an effective preventive strategy against I/R injury of an elderly liver. Their findings demonstrate that RIPC was superior to other preventing strategies, because the standard ischemic preconditioning method seemed to be ineffective in the case of old liver. Furthermore it was found that RIPC is better in promoting protective signalling, VEGF is the key mediator of protective RIPC effects in elderly liver and also reduces age-related deficiencies in hepatic sinusoids.

RIPC is an inexpensive, non-invasive, easily applicable method in different clinical situations. This method could be applied in patients undergoing liver surgery, therefore the examination of the effect of different RIPC protocols may serve further information in better understanding the pathophysiology of the process and finding the optimal way and the most effective method for clinical application to diminish the damage caused by I/R injury.

■ Conclusions

Regarding the hemodynamic and microcirculatory alterations, both early and late remote preconditioning protocols had

beneficial effect. However, the histopathological findings were paradox. Based on our results it cannot be determined which RIPC protocol provides more protection against ischemia-reperfusion injury in this rat model.

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■ Acknowledgement

The technical and laboratory staff, Department of Operative Techniques and Surgical Research, Faculty of Medicine, University of Debrecen.

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Received: Mar 12, 2018

Review: May 10, 2018

Accepted: June 09, 2018

Conflict of interest: none

Financial source: Bridging Fund, Faculty of Medicine, University of Debrecen

¹Research performed at Department of Operative Techniques and Surgical Research, Institute of Surgery, Faculty of Medicine, University of Debrecen, Hungary.