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Intestinal ischemia-reperfusion leads to early systemic micro-rheological and multiorgan microcirculatory alterations in the rat

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Abstract.
BACKGROUND: Intestinal ischemia-reperfusion (I/R) is a potentially life-threatening situation and its pathomechanism is not fully understood yet.
OBJECTIVE: To investigate the early micro-rheological, microcirculatory and morphological consequences of intestinal I/R in a rat model.
METHODS: CD rats were anesthetized and subjected to Control ($n = 7$) or I/R ($n = 7$) groups. Left femoral artery cannulation and median laparotomy were performed. In the I/R group the superior mesenteric artery was clamped for 30 minutes. Blood samples were taken before (Base) and after the ischemia, at the 30th, 60th and 120th minutes of the reperfusion (R-30, R-60, R-120). Hematological parameters, erythrocyte deformability and aggregation were determined. On the jejunum, the liver and the right kidney laser Doppler flowmetry tests were completed. At the end of experiment histological samples were taken.
RESULTS: Hematocrit, leukocyte and platelet counts increased during the reperfusion. Erythrocyte deformability worsened versus Control. All erythrocyte aggregation index values of I/R group increased gradually. Intestinal microcirculatory blood flux units (BFU) did not recover completely after ischemia, at R-30 liver BFU values were lower, and kidney values decreased by R-120. Histology showed signs of I/R injury.
CONCLUSIONS: Micro-rheological parameters may show early and significant deterioration during the reperfusion that might contribute further to microcirculatory alterations.

Keywords: Intestinal ischemia-reperfusion, microcirculation, hemorheology

1. Introduction

Intestinal ischemia-reperfusion injury may occur due to several clinical conditions, such as acute mesenteric ischemia, trauma, cardiopulmonary disease, shock, intestinal transplant rejection, volvulus and necrotizing enterocolitis in newborns [1, 2, 12]. Mortality and morbidity remained high during the last decades [19, 39]. This is in part attributable to the lack of early diagnostic markers and the paucity in preventive and therapeutic options [14]. Better understanding of the pathomechanism of intestinal ischemia-reperfusion may contribute to new treatment strategies and thus the improvement of survival.

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During ischemia blood flow of an organ is interrupted and as a result the oxygen supply and adenosine-triphosphate (ATP) production decrease. The cells start to produce energy through an anaerobic metabolism and the accumulated lactic acid will lower the intracellular pH. The restoration of blood flow, also called reperfusion, may cause further local and remote tissue injury due to the formation of reactive oxygen species including hydroxyl radical, superoxide and peroxinitrit ions [9, 11, 20, 25, 28, 31, 37].

Oxidative stress may influence the micro-rheological properties of blood, i.e. red blood cell aggregation and deformability [4–6, 21, 22, 29]. These parameters are highly important in determining microcirculation, since enhanced aggregation and impaired deformability lead to microcirculatory deterioration [6, 13, 16, 36]. The investigation of the micro-rheological parameters along with the microcirculation may provide important information about the pathomechanism of intestinal ischemia-reperfusion injury [6, 16, 24, 33].

The aim of our study was to investigate the early effects of intestinal ischemia-reperfusion on microcirculation and histomorphology of selected intra-abdominal organs and its systemic micro-rheological consequences in a rat model. We hypothesized that intestinal ischemia-reperfusion may cause deterioration in micro-rheological and microcirculatory parameters and that these alterations are associated with each other.

2. Materials and methods

2.1. Experimental animals and study design

The experiments were approved by the University of Debrecen Committee of Animal Welfare (permission Nr.: 20/2011 UDCAW) in accordance with national and EU regulations (the Hungarian Animal Protection Act (Law XVIII/1998) and the Edict 63/2010).

Female CD outbred rats (body weight: 265.5 ± 26.7 g) were randomly divided into Control ($n = 7$) and Ischemia-reperfusion ($n = 7$) groups. Animals were housed in standard size cages under conventional conditions, received standard rat food and water ad libitum. All the experiments were performed under general anesthesia (Thiopental, 60 mg/bwkg, i.p.). As premedication animals were given atropine-sulphate (0.06 mg/bwkg, s.c.). The animals were placed on a heating pad to support maintaining body temperature.

2.2. Operative techniques and sampling protocol

After shaving and disinfecting (Betadine) the middle part of the abdomen and the left inguinal region, the left femoral artery was cannulated (BD Neoflon^{TM}, 26G) under operating microscope (Leica Wild M650). Midline laparotomy was performed and the superior mesenteric artery was gently exposed. In the Control group there were no other interventions. In the Ischemia-reperfusion (I/R) group the superior mesenteric artery was clipped atraumatically for 30 minutes and 120 minutes of reperfusion was observed afterwards.

Before the ischemia (Base), just after clip removal (I-30), at the 30th (R-30), 60th (R-60) and 120th (R-120) minute of the reperfusion microcirculatory and temperature measurements were carried out and blood samples ($\sim$0.3 ml each time, anticoagulant: 1.5 mg/ml K_3-EDTA) were taken from the cannulated artery. After samplings similar volume of physiological saline solution was given. In the Control group the same time points were used for samplings and tests. At the end of the experiments samples were taken from the small intestine, liver, kidney, pancreas and lungs for histological examinations and the animals were euthanized.

2.3. Laboratory measurements

Hematological parameters were measured by Sysmex F-800 microcell counter (TOA Medical Electronics Corp., Ltd., Japan). The test requires about 70 μl of blood. In this study hematocrit (Hct [%]), red blood cell count (RBC [x10^6/μl]), white blood cell count (WBC [x10^3/μl]) and platelet count (Plt [x10^3/μl] were analyzed.

Red blood cell aggregation was determined by Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) using light-transmittance method [15]. 20 μl of blood sample is briefly disaggregated with high shear rate (600 s^{-1}) and then the shear rate drops to 0 (M mode) or 3 s^{-1} (M1 mode). Aggregation index values are determined 5 (M 5 s, M1 5 s) or 10 seconds (M 10 s, M1 10 s) after disaggregation. Higher aggregation index values reflect enhanced aggregation [15].

For testing red blood cell deformability LoRRca MaxSis Osmoscan rotational ektacytometer (Mechatronics BV, The Netherlands) was used. 10 μl of blood was gently mixed in 2 ml of polyvinylpyrrolidone (PVP) – phosphate buffered saline (PBS) solution (viscosity: 27 mPas, osmolarity: 300 mOsm/kg, pH: ∼7.3). The suspension was injected into the device which generates shear stress (SS) from 0.3 to 30 Pa, while the laser diffraction pattern was analyzed calculating elongation index values (EI) [15]. The measurements were carried out at 37 °C. For data comparison the maximal elongation index values (EI_{max}) and the shear stress belonging to the half of it ($SS_{1/2}$) were calculated using Lineweaver-Burk analysis and the ratio of EI_{max} and $SS_{1/2}$ ($EI_{max}/SS_{1/2}$) was also compared [3].

2.4. Microcirculatory investigations

Microcirculation was monitored by laser Doppler technique (LD-01 Laser Doppler Flowmeter, Experimetria Ltd., Hungary) using standard pencil probe (Oxford Optronix Ltd., UK) on the antimesenteric surface of the jejunum, on the front surface of the liver and on the middle front surface of the right kidney. The device determines blood flux units (BFU [au]) depending on the number and velocity of the moving red blood cells in the examined tissue volume. The signal was recorded for 20 seconds and the data were analyzed offline using the average values of 10-second long, noise-free, stable periods [29].

By same measurement scheduling, a digital infrared thermometer was applied on a jejunum loop to test surface temperature, and using a rectal probe body temperature was also monitored (Experimetria Ltd., Hungary).

2.5. Histological examinations

After the last blood sampling, tissue samples were taken from the small intestine, the liver, the pancreas, the right kidney and the lungs for histological examination. The samples were fixed in 5% formaldehyde-solution, dehydrated in a graded series of alcohol, embedded in paraffin, microtomed into 3–5 μm sections and stained with hematoxylin and eosin (H&E).

2.6. Statistical analysis

Data were expressed as means ± standard deviation (S.D.). For inter-group comparison Student *t*-test or Mann-Whitney rank sum tests, for intra-group comparison one-way and repeated measures ANOVA tests (Dunn's or Bonferroni's method) were applied, depending on the normality of data distribution. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hematological parameters

Figure 1 shows the alterations of selected hematological parameters. Hematocrit values of the Control group did not show important changes, while in the I/R group an increase was observed, being significantly higher during the reperfusion period (I/R vs. Control at R-30: $p < 0.001$; at R-60: $p = 0.035$; at R-120: $p = 0.001$ and vs. Base; at R-30: $p < 0.001$; at R-60: $p < 0.001$). Red blood cell count showed similar changes: in the I/R group it was elevated during the reperfusion (I/R vs. Control at R-30: $p < 0.001$ and vs. Base at R-30: $p = 0.005$; at R-60: $p = 0.021$; at R-120: $p = 0.014$).

White blood cell count in the Control group increased and remained elevated during the ischemia and reperfusion period, as well as in the I/R group, however it was higher in the I/R group at the 60th and 120th minutes of the reperfusion period (I/R vs. Base at R-60: $p = 0.003$; at R-120: $p = 0.007$). Platelet count increased during the ischemia and in the first hour of the reperfusion in the Control and I/R group as well, but there was no significant difference between the two groups.

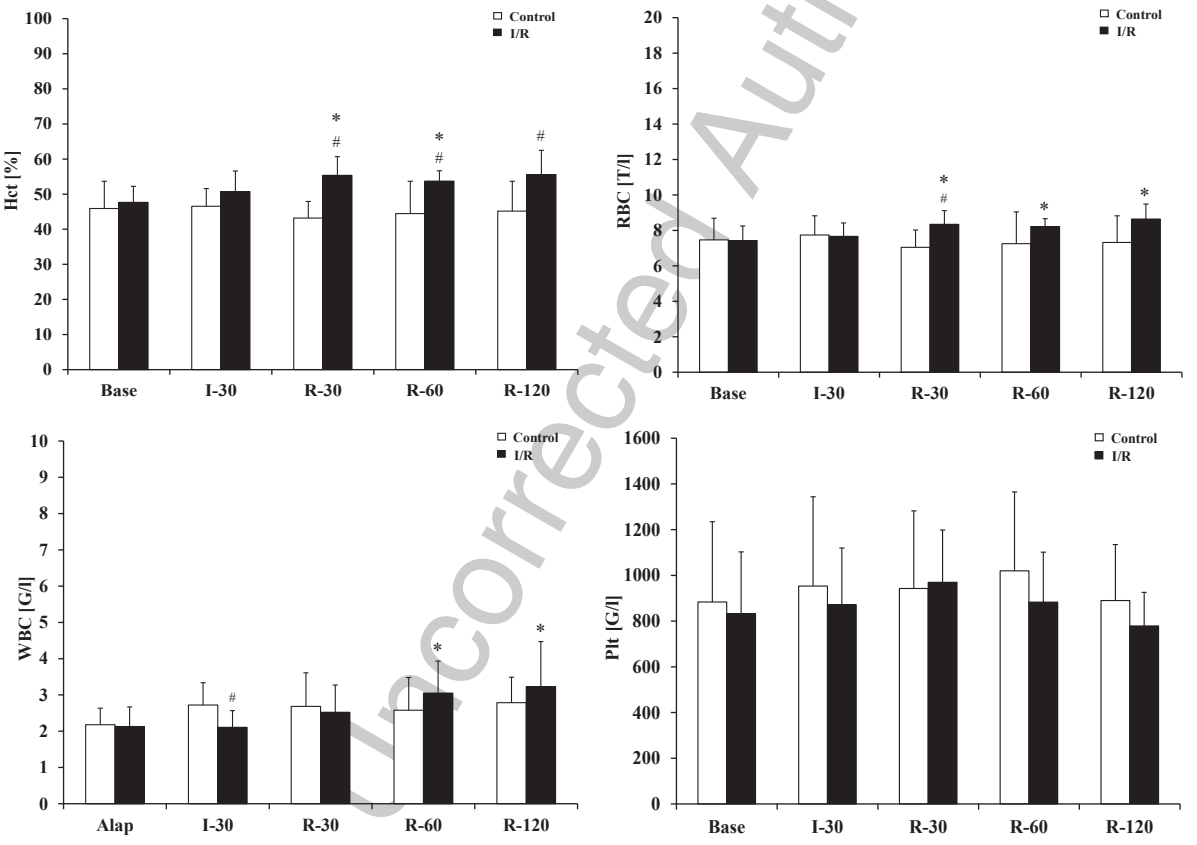

Fig. 1. Changes of hematocrit (Hct), red blood cell count (RBC), white blood cell count (WBC) and platelet count (Plt) in Control and Ischemia-reperfusion (I/R) groups. Base = before ischemia; I-30 = the end of the 30-minute ischemia; R-30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means ± S.D.; $^{*}p < 0.05$ vs. Base; $^{\#}p < 0.05$ vs. Control.

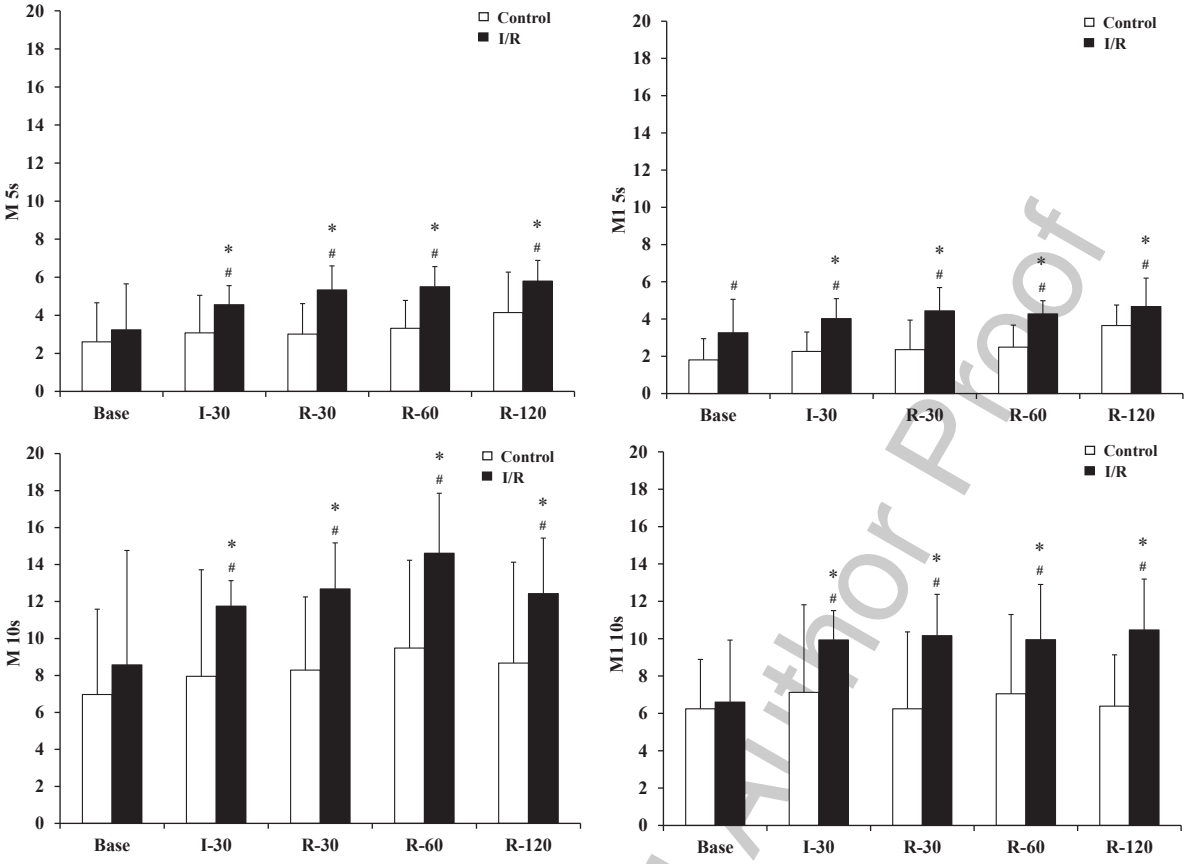

Fig. 2. Changes of aggregation index M 5 s, M1 5 s, M 10 s and M1 10 s values in Control and Ischemia-reperfusion (I/R) groups. Base = before ischemia; I-30 = the end of the 30-minute ischemia; R-30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means ± S.D.; $^{*}p < 0.05$ vs. Base; $^{\#}p < 0.05$ vs. Control.

3.2. Red blood cell aggregation

Red blood cell aggregation values (Fig. 2) were significantly higher in the I/R group during the ischemia and remained elevated during the reperfusion period (e.g. M 5 s I/R vs. Control at I-30: $p = 0.004$; at R-30: $p < 0.001$; at R-60: $p < 0.001$; R-120: $p = 0.007$ and vs. Base at I-30: $p = 0.043$; at R-30: $p = 0.001$; at R-60: $p = 0.001$; at R-120: $p = 0.001$).

3.3. Red blood cell deformability

Figure 3 shows the comparative parameters of the elongation index – shear stress curves. Elongation index values at a shear stress of 3 Pa were lower in the I/R group by the end of the ischemia and the first hour of the reperfusion, being markedly decreased at the 60th minute of the reperfusion (I/R vs. Control $p = 0.018$).

The calculated EI_{max} values lowered in the I/R group. The differences were significant versus the Control values (at I-30: $p < 0.001$; at R-30: $p = 0.016$; at R-60: $p = 0.007$; at R-120: $p < 0.001$). The $SS_{1/2}$ values decreased in the I/R group by the end of the reperfusion, without reaching the significant

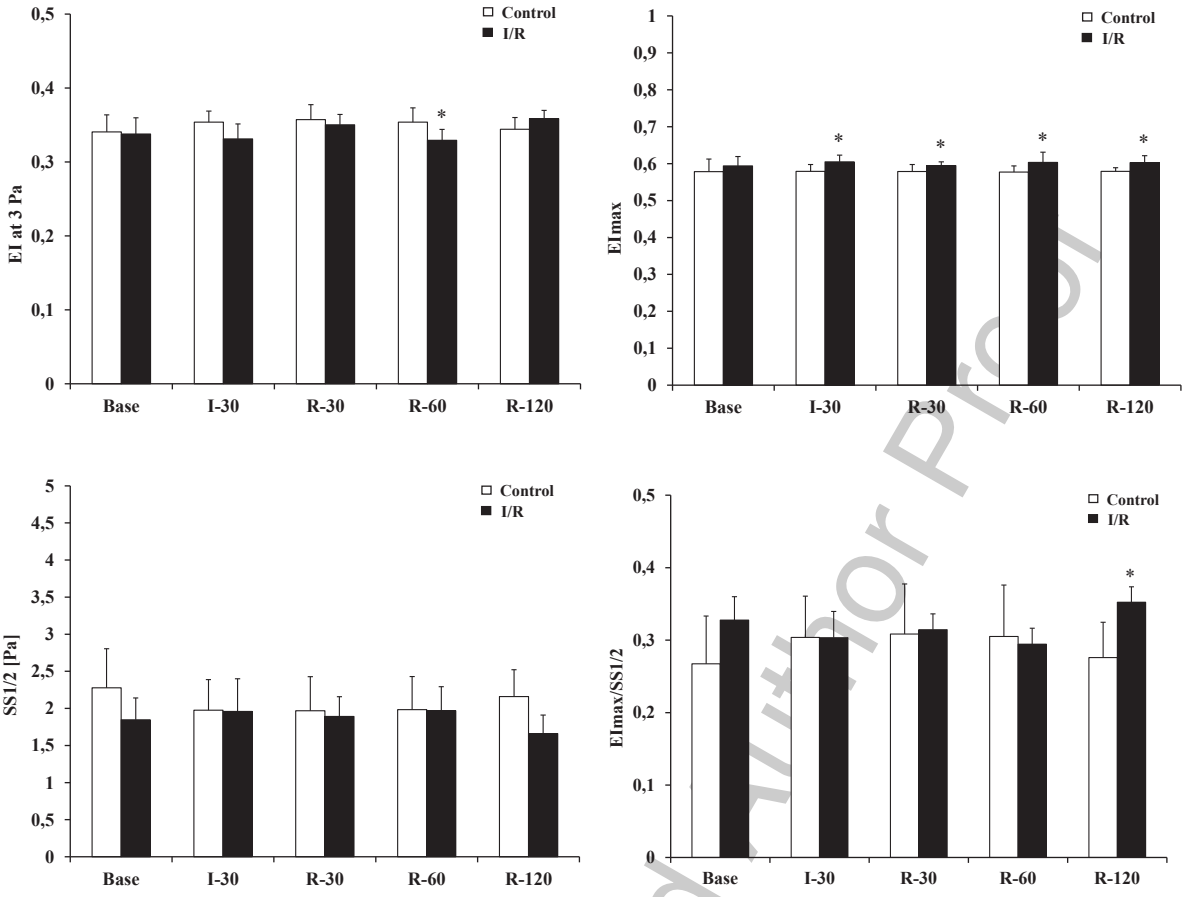

Fig. 3. Elongation index values at a shear stress of 3 Pa (EI at 3 Pa), calculated maximal elongation index values (EImax), shear stress values at half maximal elongation ($SS_{1/2}$ [Pa]) and the ratio of them ($EImax/SS_{1/2}$) in Control and Ischemia-reperfusion (I/R) groups. Base = before ischemia; I-30 = the end of the 30-minute 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 ± S.D.; $^{*}p < 0.05$ vs. Base; $^{\#}p < 0.05$ vs. Control.

level. The ratio of $EI_{max}/SS_{1/2}$ values significantly increased in the I/R group by the 120th minute of the reperfusion compared to the Control group ($p = 0.011$).

3.4. Temperature and microcirculation

In body temperature there were no significant differences between the two groups, however a slight increase could be observed by the 120th minute of the reperfusion in the I/R group. The small intestine surface temperature did not change in the Control group during the experiment, but in the I/R group a significant decrease could be seen at the end of the 30-minute ischemia (vs. Control $p = 0.002$; vs. Base $p = 0.002$).

Intestinal microcirculatory blood flux units (BFU) decreased during the ischemia (vs. Base: $p = 0.048$) but did not drop to zero, probably due to the collateral circulation of the mesentery (Figure 4). The values were higher after the clamp removal but did not normalize by the end of the reperfusion. On the liver BFU values were lower in the I/R group compared to the Control group, being the lowest at the 30th minute of the reperfusion period. The kidney microcirculatory BFU values decreased by the end of the reperfusion.

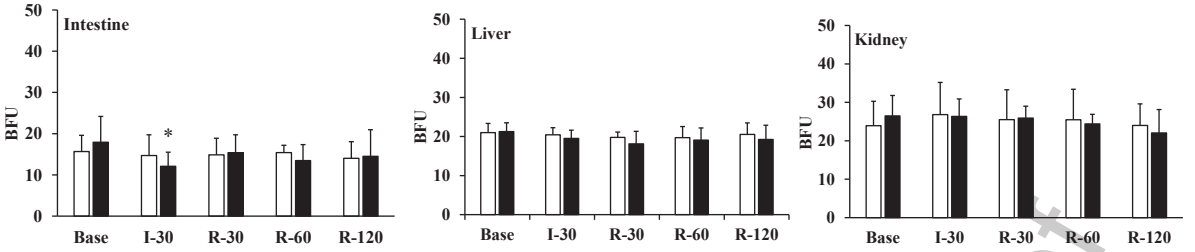

Fig. 4. Changes of blood flux units (BFU) measured on the surface of the jejunum, liver and right kidney. Base = before ischemia; I-30 = the end of the 30-minute ischemia; R-30 = the 30th minute of the reperfusion; R-60 = the 60th minute of the reperfusion; R-120 = the 120th minute of the reperfusion. Means ± S.D.; $^{*}p < 0.05$ vs. Base.

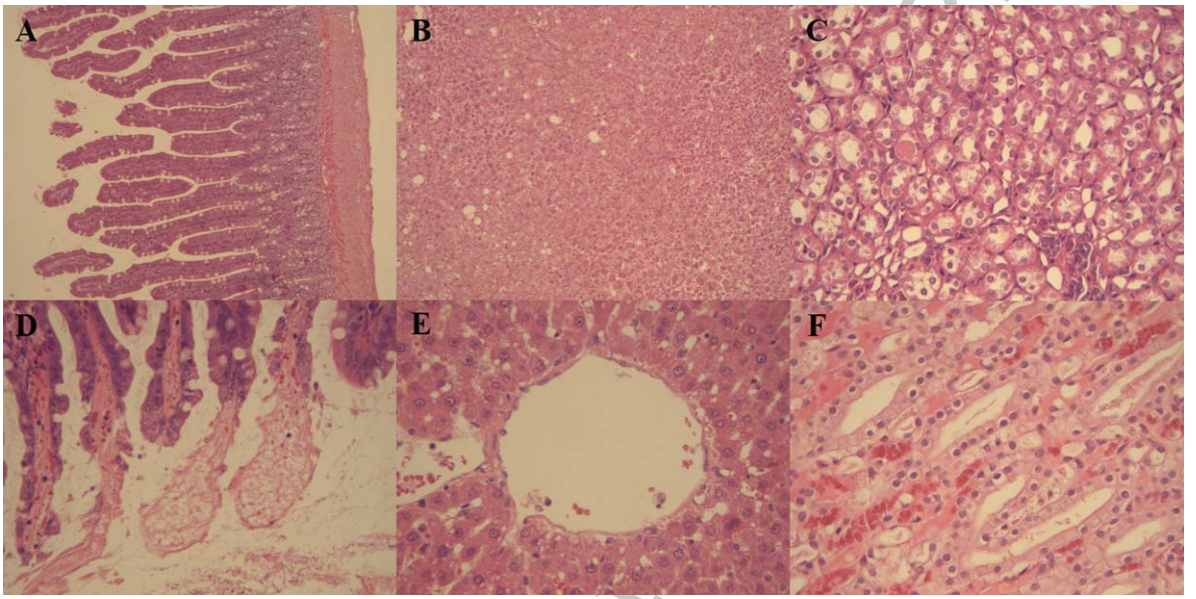

Fig. 5. Histological samples of the small intestine, liver and kidney in the Control (A, B, C) and I/R groups (D, E, F). Staining: H&E.

3.5. Histology

In the histological samples of the small intestine we could observe lamina propria hemorrhage, superficial epithelial necrosis and ulceration with minimal inflammation compared to the Control group where no damage could be detected (Fig. 5). In the liver around the central vein small drops of fat were seen and in the kidney red blood cells were observed in glomeruli and vasa recta. In the pancreas and the lungs there were no important changes.

4. Discussion

Intestinal ischemia-reperfusion may lead to life threatening complications through local and remote tissue injury. Damage occurring during the ischemia may be further exacerbated by the restoration of blood flow [7, 9, 11, 28]. Upon reperfusion oxygen is reintroduced into the tissues where it reacts with the xanthine oxidase to produce reactive oxygen free radicals [11, 23, 28, 32].

Other sources of free radicals are the nitric oxide synthases and the polymorphonuclear cells. Another event contributing to tissue injury is the so-called "no-reflow" phenomenon [8, 34, 37]. Several mechanisms may attribute to the lack of reestablishment of blood flow including intravascular thrombosis, leukocyte and thrombocyte plugging, hemoconcentration, endothelial cell swelling, vasomotor dysfunction and interstitial edema. Cell injury will lead to increased intestinal permeability, endothelial and epithelial dysfunction, interstitial edema and bacterial translocation [37]. Moreover, intestinal I/R injury is also frequently associated with liver, kidney and lungs failure [17, 27].

It is known that ischemia and reperfusion influence micro-rheological parameters of the blood. Mechanisms leading to enhanced aggregation and increased cell rigidity include free radicals (causing lipid peroxidation, hemoglobin and protein alterations), mechanical stress, changes in acid-base parameters, in lactate concentration, in osmolarity and oxygenation [4, 6, 13, 18, 35]. Interestingly, controlled reperfusion may reduce hemorheological alterations [22].

In our experiment the alterations of hematological, micro-rheological and microcirculatory parameters were investigated during 30-minute intestinal ischemia followed by 120 minutes of reperfusion. This model enables the study of early and acute changes caused by intestinal ischemia-reperfusion.

In our study, hematocrit, red blood cell count, leukocyte count significantly elevated during the reperfusion. These changes may be associated with the ischemia-reperfusion induced inflammation and acute phase reaction. These pathophysiological mechanisms may affect local and remote cells, including erythrocytes.

Our results showed that micro-rheological factors deteriorated during the ischemia and the following reperfusion. The increased red blood cell aggregation may be the consequence of free radical release and elevated fibrinogen levels due to acute injury. Decreased erythrocyte deformability may be caused by local metabolic changes and oxygen free radicals by lipid peroxidation and modified protein structure and function [6, 29, 35].

It is well documented that hemorheological parameters play an important role in determining microcirculation [7, 16, 24, 29, 36]. The worsening of micro-rheological factors was accompanied by the deterioration of microcirculation of intra-abdominal organs. It was partly due to the decreased deformability and enhanced aggregation and partly the "no reflow" phenomenon that is characteristic for tissue ischemia-reperfusion [8, 34, 37].

Intestinal blood flux units decreased during the ischemia, however the interruption of blood flow was not total. Megison et al. showed that the reduction in flow after superior mesenteric artery occlusion ranged from 44 to 97% and the individual variation was high due to the variability of collateral flow [26]. Most animal models use 30 minutes of ischemia by clamping the superior mesenteric artery, but there are several models of intestinal ischemia, e.g. the superior mesenteric artery occlusion with collateral ligation, embolization, low-flow ischemia and segmental mesenteric vascular occlusion, which all has its appropriate purpose, advantages and disadvantages [10, 12].

However, there are several limitations of the laser Doppler flowmetry as well, including drying or movement of the tissue, temperature, instability of the device, and too much pressure on the tissue, which were tried to be minimized during the measurements [30, 38].

5. Conclusion

Intestinal ischemia-reperfusion leads to significant micro-rheological deterioration and hematological alterations. The worsening of micro-rheological parameters during ischemia-reperfusion may contribute to microcirculatory disturbances of local and remote organs. These data may be useful for further studies and treatment protocols.

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The authors comply with the Ethical Guidelines for Publication in *Clinical Hemorheology and Microcirculation* as published on the IOS Press website and in Volume 63, 2016, pp. 1-2. of this journal.

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