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Age- and gender-related hemorheological alterations in intestinal ischemia-reperfusion in the rat

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ABSTRACT

Background: Intestinal ischemia-reperfusion (I/R) is a life-threatening clinical disorder. During I/R, the microrheological parameters of blood (red blood cell deformability and aggregation) worsen, which may contribute to microcirculatory deterioration. Age and gender also have a great influence on hemorheological parameters. We aimed to investigate the gender and age-related microrheological alterations during intestinal I/R.

Materials and methods: After the cannulation of the left femoral artery, median laparotomy was performed in Crl:WI rats under general anesthesia. In the young control animals there were no other interventions (female $n = 7$; male $n = 7$). In the young (female $n = 7$; male $n = 7$) and older I/R groups (female $n = 6$; male $n = 6$), the superior mesenteric artery was clipped for 30 min, and a 120-min reperfusion period was observed afterward. Blood samples were taken before and at the 30-min ischemia, in the 30th, 60th, and 120th min of the reperfusion. Hematological parameters, erythrocyte deformability, and aggregation were determined.

Results: Hematocrit increased significantly in the younger female I/R group. Red blood cell count was higher in male and older animals. In case of white blood cell count, male animals had higher values compared with females. Platelet count elevated in the younger male and older female I/R animals. Red blood cell deformability worsened, mainly in the male and older I/R groups. Enhanced erythrocyte aggregation was seen in all groups, being more expressed in the female I/R groups.

Conclusions: Microrheological parameters show gender and age-related differences during intestinal I/R. These observations have importance in the planning and evaluation of experimental data.

Introduction

Intestinal ischemia-reperfusion (I/R) is a life-threatening clinical disorder associated with several conditions, such as necrotizing enterocolitis, volvulus, trauma, mesenteric thrombi/emboli, or cardiopulmonary diseases.[1] It affects all age groups, from infants to elderly patients, and the survival rate has not improved over the last decades.[2-4] High mortality

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and morbidity is due to bacterial transclocation, systemic inflammatory response syndrome (SIRS) and remote organ failure caused by intestinal ischemia.[5]

During I/R injuries, a number of pathophysiological processes may occur, including a decreasing oxygen and adenosine-triphosphate (ATP) supply, energy production through an anaerobic metabolism, accumulation of lactic acid, lowering intracellular pH, and formation of reactive oxygen species (ROS).[6-8] It is known that these changes may cause significant alterations in the flow properties of blood, mainly in erythrocyte aggregation and deformability (microrheological parameters).[9,10] Erythrocyte aggregation is a reversible process when red blood cells (RBCs) form three-dimensional aggregates ("rouleaux" formation). Red blood cells are able to adopt a new cell shape in response to deforming forces, which is crucial for passing through capillaries that can be smaller than the cells themselves. Thus, the microrheological parameters of blood are important determinants of the microcirculation, and their investigation continues to be an interest of experimental and clinical research.[11] (Fig. 1)

I/R injuries are more prevalent in the aging population. This is in part attributable to the comorbidities that are common in elderly patients, e.g., hypertension, diabetes, or obesity. However, aging is an independent risk factor, which may cause deterioration in the cardivascular system in itself.[12,13]

Futhermore, numerous studies support the importance of gender-related differences caused by I/R injuries.[14] It has been shown that male patients are at a higher risk of cardiovascular diseases, and male gender presents a risk factor in trauma, sepsis, and other conditions. The protective effect of estrogen has been identified in several studies; however, the clinical data are still controversial. In addition, only a few studies compared the gender-related responses of microrheological parameters in I/R injuries.[14-17]

The aim of our study was to investigate the effects of intestinal I/R on systemic hemorheological parameters in a rat model. We hypothesized that during intestinal ischemia, the microrheological parameters of the blood worsen, and the degree of these changes differs in male and female, young and older rats.

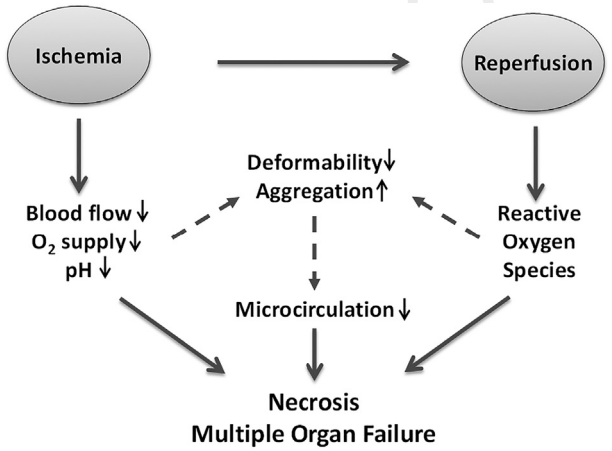

Fig. 1 – Mechanism of ischemia-repefusion injury. The deterioration of microrheological parameters may lead to the impairment of microcirculation, local, and remote organ failure.

Materials and methods

Experimental animals and study design

All experiments were performed with the approval of the University of Debrecen Committee of Animal Welfare (permission number: 20/2011 UDCAW) in accordance with national and EU regulations (the Hungarian Animal Protection Act [Law XVIII/1998] and the Directive 63/2010).

Young (4 mo old) male and female and older (18 mo old) male and female Crl:WI rats (Toxi-Coop Ltd., Hungary) were involved in the study. The animals were kept in conventional animal facility and received rat chow (Bábolna rodent-specific CRLT/N) and water ad libitum. The experiments were performed under continuous general anesthesia (thiopental, 60 mg/bwkg, i.p.).

Operative techniques and sampling protocol

Six experimental groups were formed:

I. Control young males ($n = 7$; 435.9 ± 75.2 g)
II. Control young females ($n = 7$; 281.7 ± 27.8 g)
III. Ischemia-reperfusion (I/R) young males ($n = 7$; 333.3 ± 149 g)
IV. Ischemia-reperfusion (I/R) young females ($n = 7$; 249.3 ± 25.6 g)
V. Ischemia-reperfusion (I/R) older males ($n = 6$; 622.2 ± 189.6 g)
VI. Ischemia-reperfusion (I/R) older females ($n = 6$; 548.7 ± 217 g)

In the control groups, the left inguinal region and the middle part of the abdomen were shaved and disinfected with Betadine. After isolation, an incision (~1 cm) was made on the skin above the left femoral artery. The artery was prepared and cannulated (BD Neoflon, 26 G) under operating microscope (Leica Wild M650). Midline laparotomy was performed, and the superior mesenteric artery was gently exposed by atraumatic preparation. In addition, laboratory data was used from the database of the department as old controls (females $n = 8$; males $n = 7$).

In the I/R groups, the same preparations were carried out, and the superior mesenteric artery was clamped with microvascular clip for 30 min, then a 120-min reperfusion period was observed.

Blood samples (~0.3 mL each time, anticoagulant: 1.5-mg/mL K_3-EDTA) were taken from the cannulated artery after the surgical preparations (base), at the 30th min of the ischemia, just before the removal of the clip (I-30), at the 30th (R-30), 60th (R-60), and 120th (R-120) min of the reperfusion. In the control groups, the same sampling time points were used. At the end of the experiment, the animals were euthanized.

Laboratory measurements

Hematological parameters were measured by Sysmex F-800 microcell counter (TOA Medical Electronics Corp., Ltd., Japan), which requires about 70 μL of blood. In this study, hematocrit (Hct [%]), red blood cell count (RBC [$\times 10^6$/μL]), white blood cell count (WBC [$\times 10^3$/μL]), and platelet count (Plt [$\times 10^3$/μL]) were evaluated.

For testing erythrocyte deformability, Lorca MaxSis Osmoscan device (Mechatronics BV, The Netherlands) was used at a constant temperature of 37°C. Ten microliters of blood was gently suspensed in 2 mL of isotonic polyvinyl-pyrrolidone solution (360 kDa PVP in normal phosphate buffered saline; viscosity = 27 mPa s, osmolarity = 290-300 mOsm/kg; pH ~ 7.3), and the sample was placed into the bob-cup part of the system. A laser beam is projected through it, while shear stress (SS) from 0.3 to 30 Pa is generated by the device. The laser light is diffracted by the deformed and elongated red blood cells, and a laser diffraction pattern is recorded by a video camera and analyzed by a computer that calculates elongation index (EI) values as (L − W)/(L + W), where L is the length and W is the width of the diffractogram. As a result EI-SS diagrams are made. The individual curves were analyzed by the Lineweaver–Burk method: the maximal elongation index values (EI_{max}) and the shear stress belonging to the half of it ($SS_{1/2}$) were calculated. Furthermore, the ratio of EI_{max} and $SS_{1/2}$ was also compared ($EI_{max}/SS_{1/2}$).[18,19]

Red blood cell aggregation measurements were performed by Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany). Twenty microliters of blood was used for the tests. The blood sample is sheared with high shear rate (600 s^{-1}), and then, the shear rate is reduced to 0 (M) or 3 s^{-1} (M1). Aggregation index values were determined at the fifth (M 5 s, M1 5 s) or 10th (M 10 s, M1 5 s) second of the aggregation. Higher index values reflect enhanced aggregation.[20,21]

Statistical analysis

Data are presented as mean ± standard deviation (S.D.). For intergroup comparison, Student *t* test or Mann–Whitney RS test and for intragroup comparison, one-way ANOVA tests (Dunn's or Bonferroni's method) were used depending on data distribution. A *P* value less than 0.05 was considered as statistically significant.

Results

Hematological parameters

Hematocrit (Hct [%]) increased in the younger male groups, but significant difference was not found between the control and I/R groups, whereas in the younger female I/R group, a marked increase could be observed during the reperfusion period (e.g., at R-30 *versus* control $P < 0.001$; *versus* base $P < 0.001$). In the older I/R females and males, the hematocrit values were higher compared with the younger I/R groups (*versus* younger I/R male at R-120: $P = 0.045$; Fig. 2A).

Red blood cell count (RBC [$\times 10^6$/μL]) was higher in male animals (control male *versus* control female at R-30, $P = 0.004$; at R-60, $P = 0.023$). In the younger I/R groups, a significant rise could be seen during the reperfusion in males (*versus* base at R-60, $P = 0.015$) and females (*versus* base at R-30, $P = 0.005$; at R-60, $P = 0.021$; and at R-120, $P = 0.014$; *versus* control at R-30, $P < 0.001$) as well. Higher RBC counts were measured in the older I/R groups compared with the younger same gender I/R groups (in females at I-30, $P = 0.019$), except for the R-60 values in males (Fig. 2B).

In case of the white blood cell count (WBC [$\times 10^3$/μL]), male animals had higher values compared with females. In the young I/R groups, WBC count significantly elevated by the end of the reperfusion period in both genders (*versus* base at R-120, female: $P = 0.007$; male: $P = 0.025$). In the older female and male I/R groups, the changes were of larger magnitude. During the ischemia and the reperfusion, the increased WBC count of the older I/R females was significant compared with the base values (at I-30, $P = 0.003$; at R-30, $P < 0.001$; at R-60, $P < 0.001$, and at R-120, $P < 0.001$) and the younger I/R females (at I-30, $P < 0.001$; at R-30, $P < 0.001$; at R-60, $P < 0.001$, and at R-120, $P = 0.013$). In the older male I/R group, the rise of the WBC count was only marked during the first hour of the reperfusion period (*versus* base at R-30 $P = 0.001$; at R-60 $P = 0.021$). In the younger I/R groups, the WBC count remained elevated; however, in the older I/R groups, it showed a slight decrease by the end of the reperfusion (Fig. 2C).

In platelet count (Plt [$\times 10^3$/μL]), there was an increase during the first hour of the reperfusion in the control groups and the younger female I/R group, followed by a decrease in the 120th min of the reperfusion, while in the younger male I/R group, the platelet count remained significantly higher (at R-120 *versus* base $P = 0.041$; *versus* control $P = 0.037$; *versus* female $P = 0.002$). In the older I/R groups, females showed elevated values being significant versus its base ($P = 0.046$) and compared with younger I/R females at R-120 ($P < 0.001$). In the older male I/R group, these changes could not be seen, the values were similar to the base data (Fig. 2D).

Table 1 shows the comparative data of hematological parameters in young and older control animals. Hematocrit ($P = 0.022$), red blood cell ($P = 0.017$), and white blood cell counts ($P = 0.002$) were significantly lower in the young female group compared with the older female animals. Significant differences in gender could be also observed in case of white blood cell count in young ($P < 0.001$) and older animals ($P = 0.01$).

Red blood cell deformability

The calculated EI_{max} values were the lowest in the younger male I/R group. The differences were significant versus the female I/R group (at base-R-120, $P < 0.001$), versus the control male group (at R-60, $P = 0.011$), and versus the older I/R males (at base, $P = 0.022$; at R-30, $P = 0.042$; at R-60, $P = 0.011$). In female groups, the younger I/R animals had the highest EI_{max} value from the beginning, and it remained high during the experiment. In both the younger groups, the male animals showed lower EI_{max} values compared with females. However, in the older animals, male presented higher values (at base, $P = 0.033$; Fig. 3A).

Male groups presented higher shear stress values at half EI_{max} ($SS_{1/2}$ [Pa]) compared with female groups. The highest

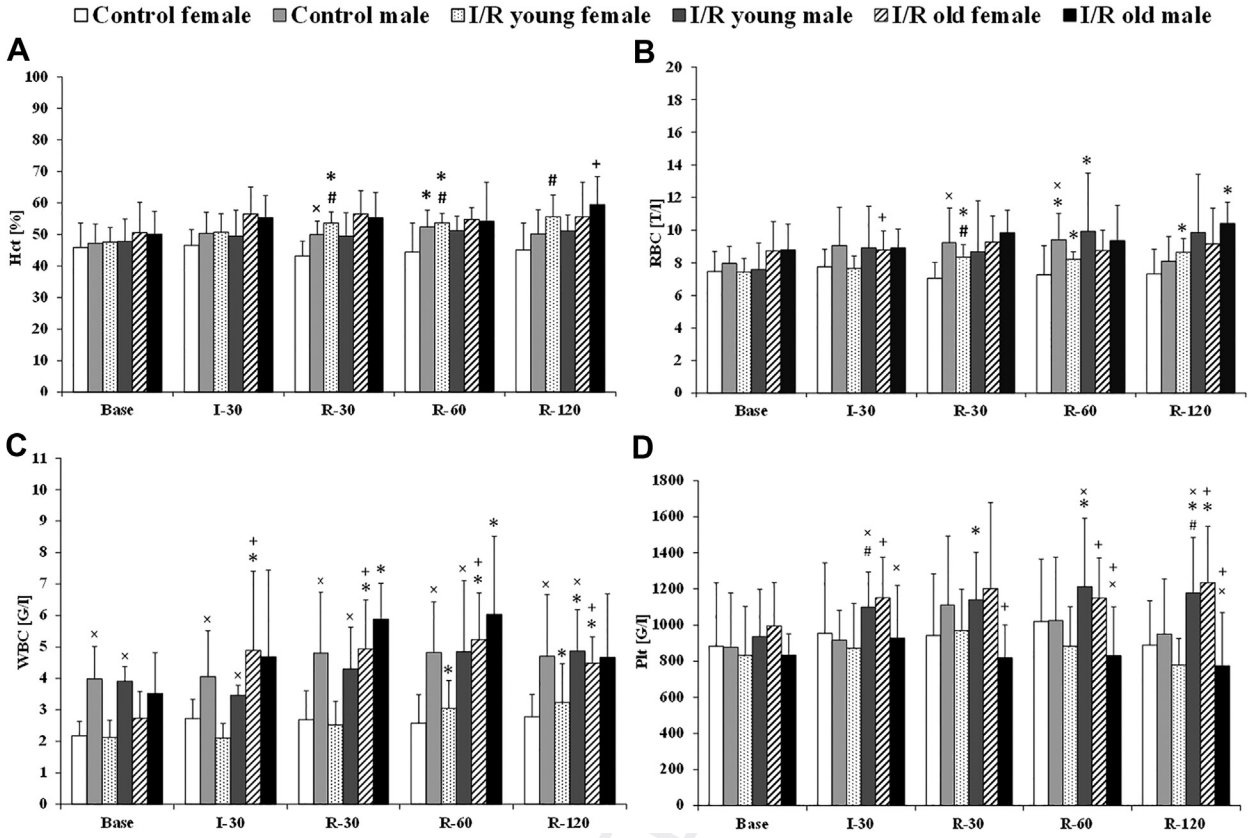

Fig. 2 – Changes of hematological parameters. (A) Hct; (B) RBC; (C) WBC; and (D) Plt in control and I/R groups. Base = before ischemia; I-30 = the end of the 30-min ischemia; R-30 = the 30th min of the reperfusion; R-60 = the 60th min of the reperfusion; R-120 = the 120th min of the reperfusion. Means ± standard deviation; $^{*}P < 0.05$ *versus* base; $^{\#}P < 0.05$ *versus* control; $^{\times}P < 0.05$ *versus* female; $^{+}P < 0.05$ *versus* I/R (same gender).

values were detected in the older male I/R group (*versus* younger male I/R group at R-30, $P = 0.025$; at R-120, $P = 0.031$). However the younger I/R males also showed elevated values compared with the control group (at I-30, $P = 0.017$; at R-60, $P = 0.006$) and the female I/R group (at I-30, $P = 0.002$; at R-30, $P = 0.02$; at R-60, $P < 0.001$; at R-120, $P = 0.003$) (Fig. 3B). The control older male group also presented more deteriorated values than the control young males ($P = 0.017$; Table 2).

Regarding the $EI_{max}/SS_{1/2}$ ratio, the same tendency was observed. Older I/R males presented the lowest values and a decrease in the younger I/R male group could be also observed (*versus* control at R-60, $P = 0.042$; *versus* I/R female at I-30, $P = 0.046$; at R-30, $P = 0.02$; at R-60, $P = 0.017$; and at R-120, $P = 0.008$) (Fig. 3C).

Red blood cell aggregation

Figure 4 and Table 2 show changes of M and M1 aggregation index values at 5 s in control and I/R groups.

Aggregation index values increased during the ischemia in the control and I/R groups as well and remained elevated during the repefusion period being markedly higher in the I/R groups (e.g., M 5s: I/R females *versus* control females at R-30, $P < 0.001$; at R-60, $P = 0.01$; at R-120, $P = 0.043$; I/R males *versus*

Table 1 – Comparative data of hematological parameters (Hct, RBC, WBC, and Plt) in young and older control groups. Q5

	Hct (%)	RBC ($\times 10^6/\mu L$)	WBC ($\times 10^3/\mu L$)	Plt ($\times 10^3/\mu L$)
Control young				
Female	45.9 ± 7.77$^{\#}$	7.47 ± 1.22$^{\#}$	2.18 ± 0.46$^{*,\#}$	883.36 ± 351.33
Male	47.23 ± 6.11	7.95 ± 1.05$^{\#}$	3.98 ± 1.03	877.86 ± 300.36
Control older				
Female	52.89 ± 16.87	8.52 ± 2.96	3.11 ± 0.87*	1000.44 ± 284.28
Male	56.01 ± 14.62	9.67 ± 2.45	4.31 ± 1.81	828.44 ± 409.71

Means ± standard deviation; $^{*}P < 0.05$ *versus* male (same age); $^{\#}P < 0.05$ *versus* older (same gender).

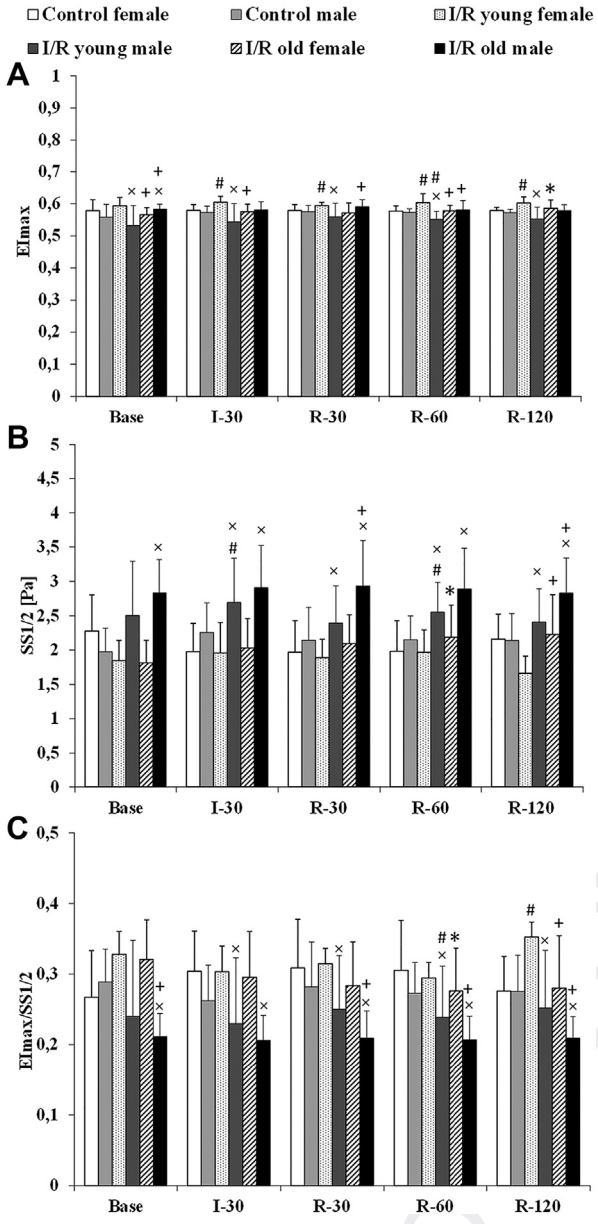

Fig. 3 – Changes of red blood cell deformability. (A) calculated maximal elongation index values (EImax); (B) shear stress values at half maximal elongation ($SS_{1/2}$ [Pa]); (C) the ratio of them ($EImax/SS_{1/2}$) in control and I/R groups. Base = before ischemia; I-30 = the end of the 30-min ischemia; R-30 = the 30th min of the reperfusion; R-60 = the 60th min of the reperfusion; R-120 = the 120th min of the reperfusion. Means ± standard deviation; $^{*}P < 0.05$ *versus* base; $^{\#}P < 0.05$ *versus* control; $^{\times}P < 0.05$ *versus* female; and $^{+}P < 0.05$ *versus* I/R (same gender).

control males at I-30, $P = 0.004$; at R-30, $P < 0.001$; at R-60, $P < 0.001$, at R-120, $P = 0.007$). In the older I/R male group, the values were lower than in the young I/R male group during the ischemia and reperfusion.

In control groups, the males had elevated aggregation index values, and significant differences could be detected between the female and male animals (e.g., M1 5 s: at base, $P < 0.005$; at I-30, $P = 0.006$; at R-30, $P = 0.041$, at R-60, $P = 0.013$ in young controls; at base, $P = 0.01$ in older Controls).

Discussion

Intestinal injury due to I/R plays a significant role in many clinical conditions, and it is also an important cause of mortality in surgical patients.[22] Among the abdominal organs, the intestine is particularly sensitive to I/R injury.[23,24]

The microrheological parameters of blood, i.e., red blood cell aggregation and deformability have a major role in tissue perfusion; therefore, their investigation may be useful in experimental surgery and microsurgery.[25-27] Several pathophysiological processes may deteriorate these parameters, including I/R injuries. During the reperfusion, oxygen is reintroduced to the tissues, and the release of free radicals may lead to the damage of the membrane of red blood cells causing the impairment of erythrocyte deformability and aggregation.[28,29]

More and more studies suggest that gender and age have a great influence on the response to I/R injury and on hemorheological parameters as well. To test the hypothesis that aging and male gender are associated with greater susceptibility to I/R injury, we investigated the effects of intestinal I/R on systemic hematological and microrheological parameters in a rat model.

Our main findings were the following: (1) Hct increased significantly in the younger female I/R group. In the older I/R groups, the values were higher compared with the younger animals. (2) RBC count was higher in male animals versus females and older I/R animals versus younger I/R groups. (3) In case of WBC count, male animals had higher values compared with females. In the older female and male I/R groups, the changes were of larger magnitude. (4) Plt count elevated in the younger I/R animals in case of the male groups, whereas in the female groups, older animals showed higher values. (5) The impairment of red blood cell deformability was observed mainly in the male and older I/R groups. (6) Enhanced erythrocyte aggregation was seen in all groups, being more expressed in the female I/R groups.

The elevated hematocrit, red blood cell, white blood cell, and platelet levels are the signs of acute phase reaction caused by the I/R. In the ischemic bowel, the activated white blood cells, endothelial cells, and platelets produce cytokines, resulting in the upregulation of cell adhesion molecules, which play a significant role in platelet aggregation and the formation of microthrombi in the vessels leading to the deterioration of microcirculation.[30,31] Several mechanisms, including oxidative stress, may change the rheological properties of red blood cells by causing lipid peroxidation, hemoglobin, and protein alterations. Reactive oxygen species are generated during the aging process (free radical theory of aging) and the reperfusion period, as well.[24,29,32]

Aging is a major risk factor for ischemic disorders, including stroke, ischemic heart disease, ischemic bowel disease, and many surgical procedures. I/R injuries affect mainly the elderly population, and some studies also presented evidence that the mechanism of I/R injury differs in older and younger animals.[33,34] Therefore, it should be

Table 2 – Comparative data of microrheological parameters (EImax: calculated maximal elongation index values; $SS_{1/2}$: shear stress values at half maximal elongation; M 5 s and M1 5 s: aggregation index values).

	EI_{max}	$SS_{1/2}$ (Pa)	$EI_{max}/SS_{1/2}$	M 5 s	M1 5 s
Control young					
Female	0.578 ± 0.034	2.277 ± 0.526	0.267 ± 0.065	2.6 ± 2.05	1.8 ± 1.14*
Male	0.558 ± 0.039	1.977 ± 0.342#	0.289 ± 0.046	3.4 ± 2.19	3.0 ± 1.59
Control older					
Female	0.571 ± 0.009	2.234 ± 0.471	0.265 ± 0.046	1.76 ± 1.07*	1.35 ± 1.11*
Male	0.573 ± 0.026	2.342 ± 0.410	0.251 ± 0.039	2.94 ± 1.42	1.85 ± 1.22

Means ± standard deviation; *P < 0.05 *versus* male (same age); #P < 0.05 *versus* older (same gender).

desirable to chose older animals in I/R models; however, usually younger animals are used in these experiments because of several reasons, such as greater availibility and lower cost. The average life expectancy of laboratory rats is 2.5-3 y. If we would like to correlate rat ages with human ages, it is important to take into consideration that relative ages are different depending on the stage of life. For example, a 4-month-old rat is considered a 10-year-old human, whereas 18 mo of rat age equals to 45 human years.[35] According to the free radical theory of aging, the process of aging is (partly) due to the oxidative stress causing damage to biologically important targets.[32] Age-related changes in different organs after I/R have been demonstrated in several studies, e.g., Azhar *et al.* found that the extent of damage caused by I/R is greater in the hearts of old mice than in the young adult mouse heart. Kusaka *et al.* showed that aged rats experience more severe reperfusion-induced injury, and Shah *et al.* observed that aged mice are more susceptible to mortality due to intestinal I/R.[12,36,37] Aging is also associated with a deterioration of the hemorheological parameters: increased blood and plasma viscosity, enhanced RBC aggregation, and impaired RBC deformability.[38,39]

Hemorheological differences regarding gender were observed in many studies in humans, too. Compared with male blood, female blood has lower viscosity, hematocrit and red blood cell aggregation, and higher erythrocyte deformability.[40-43] Furthermore, it has been revealed that the age distribution of erythrocytes is significantly different in females and males: the female blood has more young RBCs than male blood. It has been demonstrated that "old" RBCs compared with "young" RBCs have increased mechanical fragility, rigidity, and elevated aggregability.[42] These properties of the blood may contribute to the higher risk for the development of cardiovascular diseases in males.

It has been also shown that estrogen has a protective effect against I/R injury in heart, brain, and limb. The mechanisms underlying this effect are quite complex: estrogens are involved in gene transcription and intracellular signaling, have anti-inflammatory and anti-oxidant activities, and take part in vasoregulation as well.[44-46] However, some studies found that estrogen replacement therapy increased the cardiovascular incidences in women.[47] This discrepancy underlines the importance of further research in this field.

Any surgical procedure or disorder that involves the interruption of blood flow to an organ or tissue along with the restoration of blood supply can result in I/R injury, and it may lead to local and remote tissue damage. The investigation of

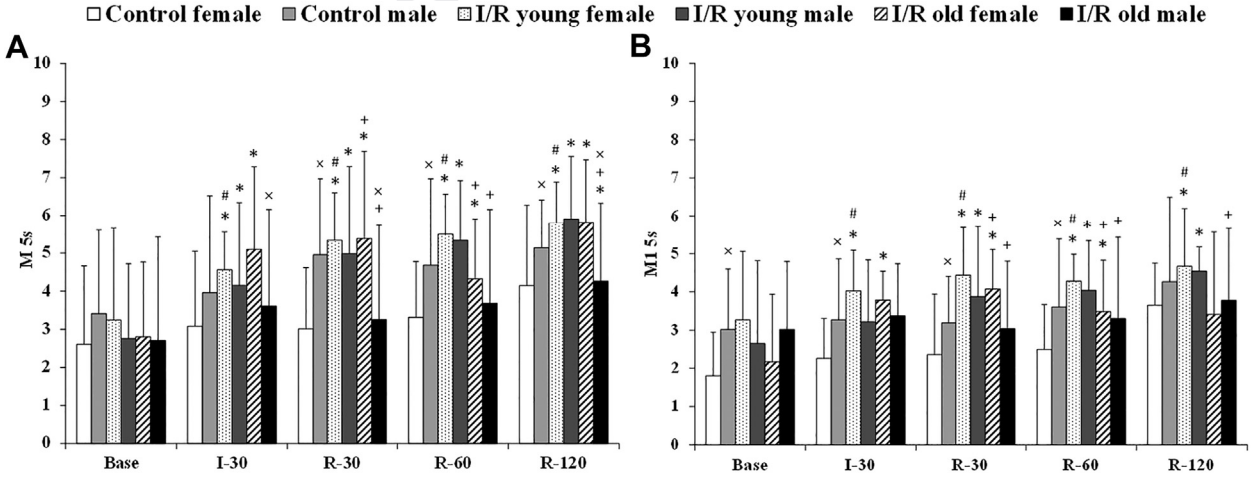

Fig. 4 – Changes of erythrocyte aggregation. M 5 s (A) and M1 5 s (B) values in control and I/R groups. Base = before ischemia; I-30 = the end of the 30-min ischemia; R-30 = the 30th min of the reperfusion; R-60 = the 60th min of the reperfusion; R-120 = the 120th min of the reperfusion. Means ± standard deviation; *P < 0.05 *versus* base; #P < 0.05 *versus* control; ×P < 0.05 *versus* female; and +P < 0.05 *versus* I/R (same gender).

the microrheological parameters may provide important information about the pathomechanism of intestinal I/R injury and potential therapeutic options. Furthermore, our study draws the attention of physicians that elderly patient may suffer more severe injuries.

Some limitations of the study have to be taken into consideration. First of all, the amount of blood samples we could take from the animals limits the number of parameters we could measure. In addition, regarding the experimental protocol, we could not perform a longer follow-up of the animals. Other issues are the interspecies differences and extrapolation of data, which is a natural limitation of every animal study. During the whole length of the observation period, we followed the young control animals, and from the older control animals, only base samples were measured. Although, in case of the base values some differences could be detected between the young and old control group (Tables 1 and 2), these differences would be the same after a 2-hour period, as well. In addition, in one of the previous studies in the department, when investigating intestinal I/R injury, the microrheological values of the control animals did not alter significantly during the experiment.[48] Therefore, taking animal welfare aspects into consideration, we tried to reduce the number of control animals, and we wished to focus on the differences of I/R between female and male or younger and older animals. However, the effect of anesthesia and samplings cannot be excluded.

Conclusions

In our present research, we demonstrated that the microrheological parameters show gender and age-related differences during intestinal I/R in rats. Erythrocyte deformability was worse in the older and male animals. Red blood cell aggregation values were heterogenous, but we can say that the most expressed rise of aggregation was observed in the older I/R females. In contrast with human data, lower deformability was not associated with enhanced aggregation in males. The cause of this difference may be the several cellular and plasmatic factors and their complex interactions that determine these parameters. These observations have great importance regarding the planning and evaluation of experiments.

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Authors' contributions: A.M. contributed to conception of the work, performed the research, and wrote the first draft of the article. Z.M., A.M., V.S., and B.T. participated in the experiments, contributed to collecting samples, acquisition, and analysis of data. K.P. and N.N. were equally responsible for manuscript preparation, critical revision of article, and final approval of the article.

Disclosure

The authors report no proprietary or commercial interest in any product or concept discussed in this article.

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