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Ischemic preconditioning and inflammatory response syndrome after reperfusion injury: an experimental model in diabetic rats

BIANCA LIANA GRIGORESCU[1)], ANCA MEDA GEORGESCU[2)], ADRIAN-DAN CIOC[1)], RALUCA-ŞTEFANIA FODOR[3)], OVIDIU SIMION COTOI[4)], PAL FODOR[5)], SANDA MARIA COPOTOIU[2)], LEONARD AZAMFIREI[3)]

[1)]*Department of Anesthesiology and Intensive Care, Emergency County Hospital, Tirgu Mures, Romania*
[2)]*Department of Infectious Diseases, University of Medicine and Pharmacy of Tirgu Mures, Romania*
[3)]*Department of Anesthesiology and Intensive Care, University of Medicine and Pharmacy of Tirgu Mures, Romania*
[4)]*Department of Pathophysiology, University of Medicine and Pharmacy of Tirgu Mures, Romania*
[5)]*Department of Orthopedics and Traumatology, Emergency County Hospital, Tirgu Mures, Romania*

Abstract

Quantification of local ischemia and inflammatory response syndrome correlated with histological changes associated with ischemia-reperfusion injury (IRI) after revascularization techniques. We included 12 adult male Wistar rats, aged eight weeks that were randomly divided into two groups. The first group acted as the control and at the second group, we induced diabetes by intraperitoneal streptozotocin administration (60 mg/kg). After eight weeks, the rats were subject to ischemic preconditioning for 10 minutes at three regular intervals. Twenty-four hours post-preconditioning, both groups were subject to ischemia for 20 minutes, followed by 30 minutes of reperfusion. Oxygen extraction was higher in Group 1, the arterio-venous CO_2 gradient was higher in the control group, but not significant. The lactate production was higher in Group 1. The second group had a higher Na^+ and also a significant difference in K^+ values. Receptor for Advanced Glycation End (RAGE) values were higher in the second group but with no significant difference ($RAGE_1$=0.32 ng/mL *versus* $RAGE_2$=0.40 ng/mL). The muscle samples from the control group displayed significant rhabdomyolysis, damage to the nucleus, while the preconditioned group showed almost normal morphological characteristics. The lungs and kidneys were most damaged in the control group, with damage expressed as thickened alveolar septa, neutrophil infiltrates, eosinophilic precipitates in the proximal convolute tubule. Ischemic preconditioning significantly attenuates the ischemic reperfusion injury.

Keywords: ischemic preconditioning, diabetes, RAGE, ischemia-reperfusion injury.

Introduction

The treatment of choice in critical limb ischemia is the peripheral revascularization, using endoluminal or surgical approach. Regardless of the chosen technique, the revascularization includes exposure of limb ischemia followed by a period of reperfusion (I/R). Sudden blood flow cease in a certain territory is accompanied by changes in the surrounding tissues, both cellular and biochemical, with severity directly proportional to the duration and degree of ischemia. The main change is represented by the prevalence of anaerobic metabolism, with decreasing cellular pH, increased influx of Na^+ and Ca^{2+}, depletion of ATP deposits and increase mitochondria permeability transition pore. These changes may be reversible upon restoration of blood flow (reperfusion), but is accompanied by the emergence of a systemic inflammatory response, which can aggravate injuries induced by ischemia, and eventually leading to multiple organ dysfunctions [1–3]. The association of other cardiovascular diseases, diabetes and chronic respiratory pathology worsens the *ad vitam* prognosis of these patients.

Ischemic preconditioning (IP) involves the application of short ischemia-reperfusion (I-R) cycles, prior to induction of long-term ischemia and confers long-lasting protective effect against ischemia-reperfusion injury (IRI).

The objective of this study was to quantify local ischemia and inflammatory response syndrome correlated with histological changes associated with IRI after revascularization techniques.

Materials and Methods

Our study included 12 male Wistar rats, aged eight weeks, weighing 350–460 g, randomly divided into two groups: control Group 1 (n=6), and Group 2 (n=6), in which diabetes was induced by injection of streptozotocin (Streptozotocin Mixed Anomers, Sigma Aldrich, Canada), 60 mg/kg i.p. [4, 5].

All experimental procedures with animals followed the international recommendations for the use and care of animals and all experimental protocols were approved by the Ethics Commission of the University of Medicine and Pharmacy of Tîrgu Mureş, Romania. The animals were acclimatized to the usual laboratory conditions 14 days before the experiment in cages with circadian rhythm of light and a stable temperature of 23^0C. During the experiment, the rats were fed with standard laboratory rodent food and water *ad libitum* provided by "Cantacuzino" National Institute of Research and Development for Microbiology and Immunology, Bucharest, Romania.

Diabetes was highlighted by serial measurements of

fasting glucose from day 4 after administration of streptozotocin and by clinical signs (polyuria, polyphagia, polydipsia, weight loss). The cut-off values of glucose were 15 mmol/dL.

Eight weeks after onset of diabetes, after fasting for 12 hours, Group 2 was subjected to ischemic preconditioning in general anesthesia with ketamine–xylazine (50 mg/kg ketamine, 10 mg/kg xylazine) intraperitoneally administered. The involved technique consisted of applying a tourniquet to the root of left lower limb (three cycles of 10 minutes of ischemia/reperfusion), according to the validated protocols. To avoid femoral shaft thrombosis subjects received heparin sodium 250 IU/kg prior clamping.

Twenty-four hours after preconditioning, in general anesthesia with ketamine–xylazine, both groups were subjected to acute ischemia for 20 minutes followed by reperfusion for 30 minutes. Biological samples harvested by arterial and venous puncture of the left iliac artery and vein, performed after declamping the blood vessels.

After euthanasia of both groups biopsy samples of striated muscle, liver, kidney and lung were obtained. Blood gasometry was measured by using an ABL800 Basic radiometer (Copenhagen, Denmark).

The obtained serums were centrifuged at 2000 rpm for three minutes and stored at -20^{0}C, serum levels of receptor for advanced glycation end (RAGE) were measured by Enzyme Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (Abcam).

Biopsy samples were processed by standard Hematoxylin–Eosin (HE) staining and evaluated under optical microscopy using Nikon Eclipse E800 (Japan) with the objective 2×, 4×, 10×, 20×, 40×.

The results were expressed as the mean values of the data obtained and processed using SPSS version 20 (USA, California) using *t*-test for paired data. The confidence intervals were set at a 95% with a significant $p<0.05$.

Results

Analyzed parameters aimed to quantify local and general changes associated with IRI. Tissue oxygen extraction, arterio-venous gradient of CO_2, the concentration of lactate and ionic imbalances induced by local ischemia and systemic inflammatory response syndrome by dosing serum RAGE, correlated with histological changes.

Hypoxia induced by ischemia

In the conditions of ischemia installation, tissue oxygen supply decreases, which subsequently increases oxygen tissue extraction, and the shift to anaerobic metabolism takes place. This results in lactate accumulation, aerobic CO_2 production decline and its increased anaerobic turnover.

Tissue oxygen extraction, quantified as the arterial-venous difference of oxygen partial pressure was significantly higher in the control group [$P_{1(av)}O_2$=18.32 mmHg *versus* $P_{2(av)}O_2$=5.15 mmHg, p=0.047] compared to Group 2 (ischemic preconditioned).

The variation of arteriovenous CO_2 gradient between the two groups was not statistically significant. The average value of $P_{1(av)}$=46.52 mmHg CO_2 was compared with $P_{2(av)}$=41.28 mmHg (p=0.175).

Lactate follows the same trend as tissue oxygen extraction, and is significantly higher in Group 1 (lactate$_1$ 20.33 mg/dL *versus* lactate$_2$ 13.33 mg/dL, p=0.004).

Changes in ionic balance

Changes in sodium levels have double etiology, *i.e.*, ischemic and due to reperfusion. Sodium distortion being assigned to the Na^+–H^+ cotransporter inhibition and intracellular Ca^{2+} changes, while hyperkalemia is associated with cytolysis and metabolic acidosis.

In our study, serum sodium levels showed significantly elevated mean values in Group 2 *versus* Group 1 (Na_1 146.17 mEq/L, Na_2 151.83 mEq/L, p=0.011). The studied groups did not develop hyperkalemia. There was no statistically significant difference between mean serum values of potassium (K_1 3.42 mEq/L *versus* K_2 2.88 mEq/L, p=0.008).

RAGE variation

Receptor for advanced glycation end products (RAGE) is an immunoglobulin present on the cell surface. It has different glycosylated ligands and is responsible for amplifying the inflammatory response associated with different pathological conditions including diabetes, thus contributing to disease progression. RAGE values are elevated in the presence of oxidative stress in the tissue or basal lateral membranes of type I pneumocytes in acute lung injury.

RAGE values were determined from the serum were increased for Group 2 (mean $RAGE_2$ 0.40 ng/mL) *versus* Group 1 (mean $RAGE_1$ 0.32 ng/mL), p=0.08, but without statistical significance between the two groups.

Local and systemic morphological changes associated to IRI

Local changes refer to the degree of muscle breakdown after acute ischemic event in healthy rats and diabetic rats. Ischemic preconditioning and systemic changes highlight the presence and the degree of inflammatory syndrome in the lungs, kidneys and liver after ischemia reperfusion injury.

Changes in skeletal muscles

Microscopic examination of the muscles from the control group (using HE staining) with various objectives, highlighted muscle injury characterized by severe rhabdomyolysis with no striations and lack of the nucleus ("ghostly" appearance of muscle fibers) or moderate rhabdomyolysis with striations disappearance and outbreaks with hypereosinophilic sarcoplasm (Figures 1 and 2). In diabetic rats, ischemic preconditioning injuries were mild showing a preserved structure, with striations and many nuclei in the sarcoplasm, with characteristic willing on its periphery, just under the sarcolemma (Figure 3). Atheroma, secondary to diabetes, was revealed by the presence of atheromatous plaques at a section of the muscle containing the vessel.

Changes in lungs, kidneys and liver

Lung parenchyma changes were most obvious in the

control group. Microscopic examination revealed lesions of variable intensity, diffuse alveolar damage with thickened septa and neutrophilic infiltrates, also at the level of interstitium, or outbreaks of foamy macrophages in the alveoli and peribronchial areas (Figures 4–6). In Group 2, lesions were minimal, with apparently normal lung, alveolar septa of normal thickness and absence of cellular infiltrate, exudates or fibrin deposits, but with vascular stasis (Figure 7). Liver damage was absent in both groups.

Renal lesions were expressed in the control group, because of rhabdomyolysis, highlighting the eosinophilic precipitate in the proximal convoluted tubules with finely granular or multivacuolar appearance. In the diabetic rats, due to ischemic preconditioning appearance is near normal.

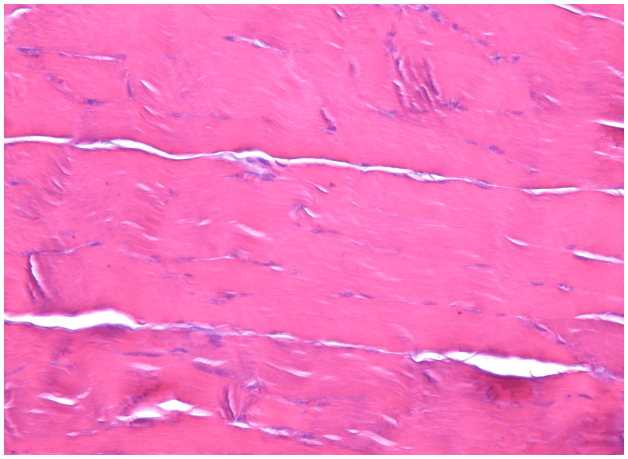

Figure 1 – ***Muscle injury characterized by severe rhabdomyolysis with striations disappearance and outbreaks with hypereosinophilic sarcoplasm. HE staining, ×200.***

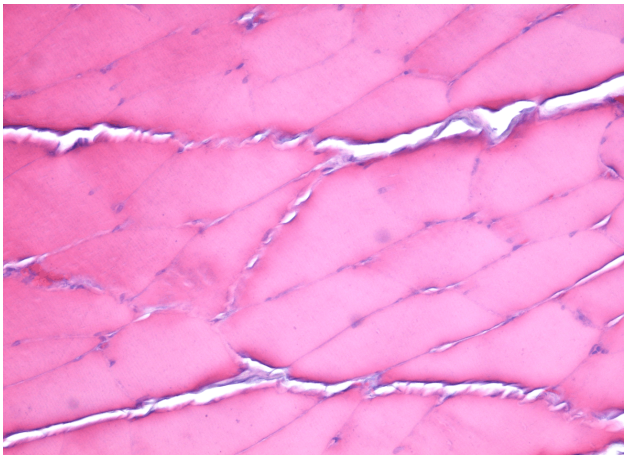

Figure 2 – ***Severe rhabdomyolysis with no striations and lack of the nucleus – "ghostly" appearance of muscle fibers. HE staining, ×200.***

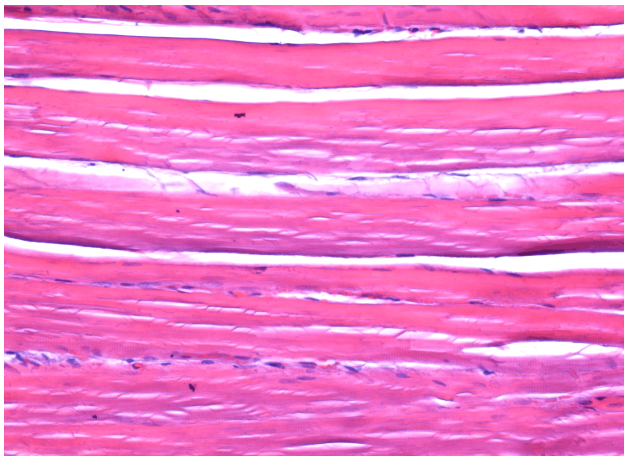

Figure 3 – ***Preserved structure with striations and many nuclei in the sarcoplasm, with characteristic willing on its periphery, just under sarcolemma. HE staining, ×200.***

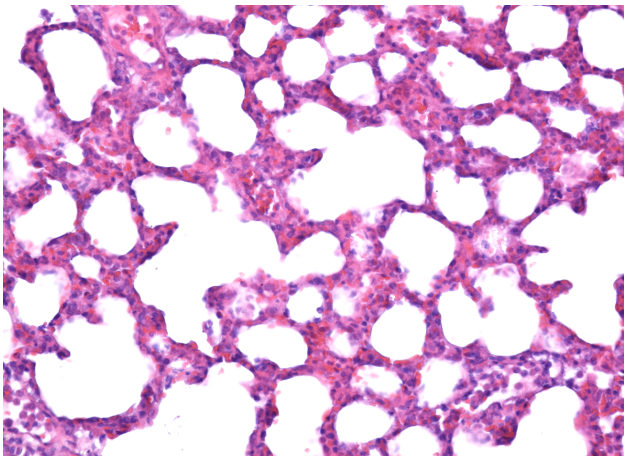

Figure 4 – ***Diffuse alveolar damage with thickened septa and neutrophilic infiltrates. HE staining, ×200.***

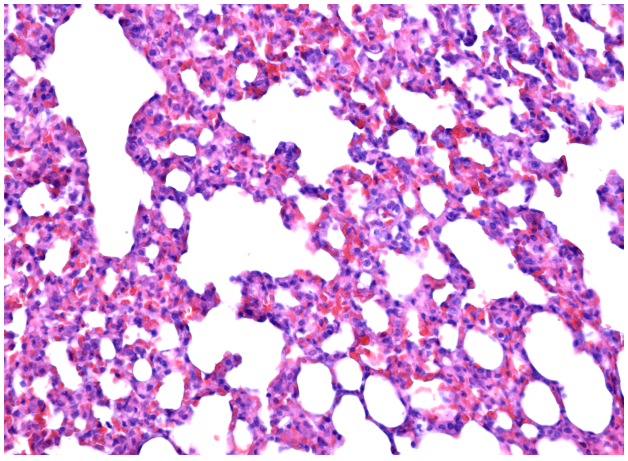

Figure 5 – ***Erythrocytes and neutrophilic infiltrates in the interstitium and in the level of thickened alveolar septa. HE staining, ×200.***

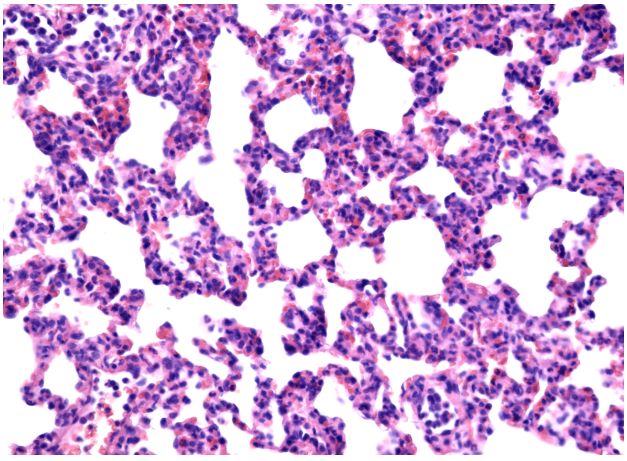

Figure 6 – ***Diffuse alveolar damage with thickened septa and neutrophilic infiltrates also at the level of interstitium or outbreaks of foamy macrophages in the alveoli and peribronchial areas. HE staining, ×200.***

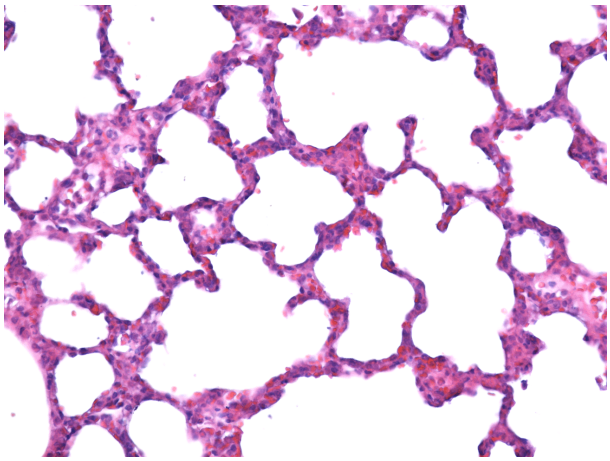

Figure 7 – ***Apparently normal lung, alveolar septa of normal thickness and absence of cellular infiltrate, exudates or fibrin deposits, but vascular stasis. HE staining, ×200.***

Discussion

Ischemia is defined by the absence of blood flow, implicit oxygen supply, and nutrients in a certain territory. Depending on the circumstances and rapidity of installation and the duration to restore blood flow, lesions secondary to ischemia can be reversible or irreversible, causing dysfunction, injury or cell death [6, 7].

The main mechanism of hypoxic or ischemic cellular injury occurrence is the alteration of oxidative phosphorylation and ATP-deposits. Reduction in the partial pressure of oxygen causes oxidative phosphorylation cessation and reduces production of ATP [8]. During ischemia, the imbalance between oxygen supply and consumption determines the cleavage of ATP with the release of adenosine, which causes vasodilatation favoring tissue oxygen extraction with protective effect against ischemic injury.

Our study supports those findings. The tissue oxygen extraction (quantified as an arterial-venous oxygen difference in blood) in the control group was higher than in Group 2 (diabetes, ischemic preconditioning). Ischemic preconditioning by short periods of ischemia induction and reperfusion, improved blood flow to the muscles, meanwhile increasing adenosine levels and the number of K^+-ATP channels, stimulating mitochondrial biogenesis and relieving tissue oxygen demand [9, 10].

Induction of short I-R periods before long lasting ischemia induces arteriogenesis, angiogenesis, vascular remodeling, and thus compensating low oxygen demand [11]. Depletion of ATP deposits is followed by efflux of K^+ ions, Ca^{2+} influx, Na^+ ions with water, and development of cellular edema. Under persistent ischemia, when the capacity of compensatory mechanisms is exceeded, the irreversible changes occur with the installation of necrosis [12]. Decreased oxygen supply is followed by anaerobic glycolysis with decreasing pH and accumulation of lactic acid in the tissues [13]. In our study, the mean value of serum lactate is significantly higher in the control group, compared to diabetic rats ischemic preconditioning.

Elevated lactate levels in arterial blood can be used as a marker of anaerobic metabolism, but also may be a consequence of functional impairment of pyruvate dehydrogenase, secondary inflammatory syndrome associated IRI [14, 15]. Improving oxygen supply for ischemic preconditioning helps to increase lactate removal and its mitochondrial influx [9]. In our study, average value in preconditioned rats was within normal limits (normal values 5–14 mg/dL). Randal & Cohen showed that secondary tissue hypoxia, decreased global consumption of oxygen is associated with reduced aerobic and anaerobic production of CO_2. Therefore, it's reduced buffering capacity to lower pH and accumulation H^+. It demonstrates that the production of CO_2 should be smaller than O_2 consumption [16]. No statistically significant difference between the groups studied, although higher mean values of arteriovenous difference of partial pressure of CO_2 were in the control group [$P_{1(av)}CO_2$=46.52 mmHg compared to $P_{2(av)}CO_2$=41.28 mmHg]. This is explained by the presence of lung injury due to reperfusion injury (diffuse alveolar damage, thickened septa with neutrophils in the septa and interstitial) with altered gas exchange.

Ionic imbalances are consistent with highlighted tissue changes on direct microscopic examination, *i.e.*, rhabdomyolysis, disappearance of striations and the presence of outbreaks with hypereosinophilic sarcoplasm. Levels of serum K^+ increased mostly in the control group, as the consequence of rhabdomyolysis and metabolic acidosis secondary to elevated lactate values. In terms of serum sodium, the diabetic rats presented significantly elevated values.

Anaerobic glycolysis and ATP degradation, buildup of H^+ with subsequent activation of Na^+/H^+ ion exchanger and Na^+ influx [6, 17]. This leads to the accumulation of Na^+ intracellularly with decreased serum values. Lazdunski *et al.* [18] highlighted the idea that reperfusion activates Na^+/K^+ reversing exchanger Na^+/Ca^{2+} exchange, while Imahashi *et al.* [19] have demonstrated a decrease in Na^+ after reperfusion. In our study, serum sodium levels have values close to normal healthy rats, but are significantly higher in diabetic rats. Although ischemia has inhibitory effects on Na^+/K^+-ATPase, we considered that hypernatremia is a consequence of dehydration associated with diabetes (polyuria) and not to the dysfunction Na^+/K^+ exchanger. Morphological changes of renal ischemia-reperfusion injury were evident in the control group, on which the presence of eosinophilic precipitate in the renal tubules (resulted by precipitation of myoglobin in acid medium) had no consequences on kidney function in the next period after revascularization. In diabetic rats, changes were minimal.

In individuals with diabetes, angiogenic response to hypoxia is attenuated, which causes long-term vascular complications. Advanced glycation end products (AGEs) are represented by modified proteins or lipids that are glycated or oxidized after contact with aldose (non-enzymatic). They are found in large amounts in the vascular wall, being involved in the emergence and development of macro- and microvascular lesions secondary to diabetes by cross-linking the molecules of the extracellular matrix and basement membrane or by binding to a receptor for

advanced glycation end products (RAGE) [20]. Belonging to a RAGE superfamily of immunoglobulin of cell surface molecules, gives possibility to participate in the host responses to environmental changes such as cell adhesion, more than as a scavenger modified peptide [21, 22]. RAGE activation increases endothelial permeability for various macromolecules, especially in the vessels in individuals with diabetes. Our study has highlighted the link between vascular lesions induced by the presence of diabetes and serum amount of RAGE, demonstrating an average of about 0.40 ng/mL, which is increased compared to baseline values (<0.17 ng/mL) of diabetic rats. This increase is primarily due to macro- and microvascular arterial arteriopathy, secondary to diabetes, argument that is also supported by pathology examination of intravascular atherosclerotic plaque in the striated muscle. In diabetic rats with ischemic preconditioning microscopic examinations of different tissue, fragments revealed no pathological changes. RAGE was originally considered as a marker of vascular endothelial damage associated with diabetes mellitus but in the last decade, it has been pointed out that RAGE is present in various cells and tissues in varying amounts associated with certain pathological conditions: amyloidosis, renal failure, oxidative stress, and in the inflammatory response of various etiologies, by activation of numerous cytokines and adhesion molecules involved in the induction and maintenance thereof [20]. Mukherjee *et al.* (2008) showed that in lung, RAGE is localized to the baso-lateral membrane of type I lung cells and is involved in amplifying the immune response in various diseases and contributing to their progression [23], including in acute lung injury/acute respiratory distress syndrome (ALI/ARDS). In our study, the presence of soluble RAGE in serum, in the control group, is associated with reperfusion lung injury, highlighted by microscopic diffuse alveolar damage, thickened septa with neutrophilic infiltrates present at their level and in interstitium. Serum values were lower in diabetic rats ($RAGE_1$ 0.40 ng/mL) but not statistically significant. The increase has been regarded as a marker of systemic inflammatory response syndrome and oxidative stress secondary to acute ischemia of the lower limb.

Conclusions

Ischemic preconditioning prevents local and systemic effects of ischemia-reperfusion injury and the amplitude of inflammatory response syndrome. Induction of short I-R periods previous long lasting ischemia induces vascular remodeling and thus compensating low oxygen demand. The increased serum levels of RAGE could be a useful marker in early systemic inflammatory response syndrome induced by acute ischemia and reperfusion.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Corresponding author
Anca Meda Georgescu, Associate Professor, MD, PhD, Department of Infectious Diseases, University of Medicine and Pharmacy of Tîrgu Mureş, 48 Gheorghe Marinescu Street, 540136 Tîrgu Mureş, Romania; Phone +0744–335 657, e-mail: ancameda.georgescu@umftgm.ro

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