Effects of pharmacological and surgical interventions on ischemia reperfusion injury in bilateral acute hind limb ischemia rat model.

PhD Thesis

by

Tibor Nagy MD

Supervisor

Endre Arató MD, PhD

Head of PhD Program

Gábor Jancsó MD, PhD

University of Pécs, Faculty of Medicine

Department of Surgical Research and Techniques

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ABBREVIATIONS

ANOVA	**one-way analysis of variance**
ARDS	**acute respiratory distress syndrome**
ATP	**Adenosine triphosphate**
cAMP	**cyclic adenosine monophosphate**
cDNA	**complementary deoxyribonucleotic acid**
COX-2	**cyclooxigenase-2**
EA	**Ethacrynic acid**
ECG	**electrocardiograph**
EDTA	**Ethylene diamine tetra acetic acid**
ELISA	**Enzyme linked immunosorbent assay**
eNOS	**Endothelic nitric oxide synthase**
EtOH	**Ethanol**
GSH	**Reduced glutathione**
GST	**Glutathione S-transferase**
H_2O_2	**Hydrogen peroxide**
HE	**hematoxylin and eosin**
HO^-	**Hydroxyl anion**
IAA	**infrarenal abdominal aorta**
IL-6	**Interleukine-6**
iNOS	**inducible nitric oxide synthase**
JNK	**c-Jun N-terminal kinase**
LPS	**lipopolysaccharide**
MAPK	**Mitogen activated protein kinase**
MDA	**Malondialdehyde**
mPTP	**mitochondrial permeability transition pore**
mRNA	**messenger ribonucleotic acid**
NFkB	**Nuclear factor kappa B**
NMJ	**Neuro-muscular junction**

NO	**Nitric oxide**
O^{2-}	**Superoxide anion**
PCR	**Polymerase chain reaction**
PDE	**Phosphodiesterase**
PostC	**Postconditioning**
PPARG	**Peroxysome proliferator activated receptor gamma**
PPARGA	**PPARG agonist**
PTX	**Pentoxifylline**
RISK	**Reperfusion injury salvage kinase**
ROI	**Reactive oxygen intermediers**
ROS	**Reactive oxygen species**
SD	**standard deviation**
SEM	**standard error of mean**
-SH	**-Thiol group**
SOD	**Superoxide dismutase**
TNF-alpha	**Tumor necrosis factor-alpha**
TRIS	**Tris (hydroxymethyl) aminomethane**

1. INTRODUCTION

In patients suffering from cardiovascular diseases in many cases revascularization surgery is the only way to restore normal function, but volume and pressure overload, oxidative stress and the developing reperfusion injury frequently induce complications. In vascular surgery the acute limb ischemia is a severe, potentially life-threatening state. In this case it is necessary to perform a prompt revascularization. The duration of acute ischemia could also be serious, thus after the revascularization we always have to face reperfusion injury. The severity of reperfusion injury depends on the ischemic time, the mass of ischemic tissue and the collateral circulation of the affected tissue. In the surgical research it is of real clinical importance to reduce the extent of reperfusion injury associated pathways. [1, 2, 3] The role of oxygen free radicals in reperfusion injury is well known, accordingly to decrease of these harmful agents is very important. Catalase, superoxide dismutase (SOD), glutathione peroxidase and repair enzymes are in the first line of antioxidant protection, but recently among other antioxidant enzymes researches are focus on glutathione S-transferase (GST).

1.1. Ischemia-reperfusion injury

1.1.1. General background

Reperfusion injury is an integrated response to the restoration of blood flow after ischemia involving mechanical, extracellular and intracellular processes. It is initiated at the very early moments of reperfusion, lasting potentially for days. The modern hypothesis of the pathogenesis of reperfusion injury has been reviewed by Piper and al. [4] Patients suffering from acute arterial occlusion, it is now widely accepted that periodically reopening the occluded artery is accompanied by a reduction of the extent of necrosis and a major reduction of short- and long-term mortality. However, together with a definite protective effect on ischemic tissues, post-ischemic reperfusion may bring with it unwanted consequences that may partly counteract its beneficial effects. This phenomenon has thus been named reperfusion injury. Ischemia-reperfusion in the tissue can lead to various forms of cell death, such as apoptosis, oncosis and necrosis. [5] It seems that the occurrence of

oxidative stress related to the generation of ROS may play important role. [6] ROS can initiate an acute inflammatory response through the release of cytokines, activation of vascular endothelial cells and leukocytes with expression of cell surface adhesion molecules, and up-regulation of pro-inflammatory genes, which contribute to the onset and maintenance of inflammation. [7] When the ischemic tissue is revascularized, superoxide anion (O^{2-}) production is highly increased as a result of the activation of various enzymatic complexes. Superoxide anion dependent damages are reduced if O^{2-} is transformed to hydrogen peroxide (H2O2) by the superoxide-dismutase. However, since in the presence of Fe^{2+} or Cu^{2+}, the H_2O_2 can be transformed in hydroxyl anion (HO^-), which is more toxic than O^{2-} and H_2O_2, an increase in toxicity can occur. The overload of Ca^{2+} increases the cellular osmolarity favouring swelling (explosive swelling) of skeletal muscle cells; it can also favour the expression of proapoptotic elements from mitochondria. [8] Ca^{2+}-overload is also considered to be responsible for the opening of mitochondrial permeability transition pore (mPTP). Although, mPTP opening is strongly inhibited by acidosis during ischemia, it is favoured by ATP depletion, oxidative stress and high intramitochondrial Ca^{2+} concentrations, conditions all occurring during myocardial reperfusion. [9] Among the outcomes of reperfusion injury are included: (I.) endothelial and vascular dysfunction and the sequels of impaired arterial flow, which may concur with the 'no-reflow phenomenon'; (II.) metabolic and contractile dysfunction; (III.) arrhythmias in case of myocardial I/R; (IV.) cellular death by cellular swelling, and apoptosis. One may anticipate that effective treatment during reperfusion may reduce tissue injury. However, the complexity of mechanisms suggests that one single intervention aimed to contrast just one or two of these mechanisms may not be sufficient.

1.2. Ischemic postconditioning

To protect the heart against ischemia-reperfusion injury ischemic postconditioning is a well known method. [10] The concept of 'Ischemic postconditioning' (PostC) was first described by Vinten-Johansen's group. [27] In general, PostC can be defined as intermittent interruption of coronary flow in the very early phase of a reperfusion, which leads to

protection against reperfusion injury. The duration and number of these stuttering periods of reperfusion and ischemia has been one of the aims of early studies on this topic.

1.2.1. The possible protective mechanisms of postconditioning

Any protective strategy applied at the time of reperfusion must provide protection against the known mediators of lethal reperfusion injury, which include cellular and mitochondrial calcium overload, a burst of oxidative stress, endothelial dysfunction, and reduced nitric oxide production. The mechanisms of postconditioning are realized on passive and active way. According to the passive way in the course of gradual reperfusion PostC delays the washout of adenosine, decreases extracellular levels and the accumulation of noxious metabolites which attenuates superoxide anion generation by activation of neutrophils and endothelial cells, and activates mitochondrial KATP channels via adenosinergic G protein-coupled receptor activation. Better endothelial function increases nitric oxide (NO) release by endothelial cells, which further attenuates superoxide anion levels and both neutrophil activation and adherence to the endothelial cells. Postconditioning decreases the intracellular buildup of oxidants and calcium in cardiomyocytes, which inhibits mPTP opening, thereby inhibiting both apoptosis and necrosis. Regarding the active way possible to protect the reperfused myocardium by activating prosurvival kinase signaling pathways (reperfusion injury salvage kinase pathway - RISK). [11] Both pre- and postconditioning activate the same key pathways, which include phosphatidylinositol 3-kinase-Akt and extracellular signal–regulated kinase (ERK/p42-44) [11, 12]. Upstream may be activation of G-protein coupled receptors, and the many downstream events include key phosphorylations of endothelial nitric oxide synthase (eNOS) and inhibition of the apoptosis promoters. Protective pathways activated by postconditioning appear to converge on the mitochondria, in particular the mitochondrial permeability transition pore. This opens during the first few minutes of reperfusion, in response to mitochondrial calcium overload, oxidative stress, and adenosine triphosphate depletion [13]. Postconditioning protect the heart through the inhibition of mitochondrial permeability transition pore opening. [14] Taken together postconditioning influence a variety of endogenous mechanisms that operate at numerous levels and target a broad range of pathological mechanisms.

2. AIMS

We planned to perform three major investigation.

In the ***first study*** we performed acute bilateral hind limb ischemia and reperfusion on rat animal model. In the study we investigated the effects of inhibition of - an endogenous antioxidant enzyme - Glutathione S-transferase by ethacrynic acid on ischemia reperfusion injuries and its influence on possible protecting effect of ischemic postconditioning. We examined the developing oxidative stress and inflammatory response and the histological changes in structure of skeletal muscle. We investigated various pro- and antiapoptotic signaling pathways.

The aim of the ***second study*** was to investigate the effects of novel therapies on bilateral hind limb ischemia reperfusion injury following a temporal infra-renal aortic occlusion and reperfusion. A Peroxysome proliferator activated receptor-gamma agonist (PPARGA) was applied in rats. Two major experiments were planned: time course experiment, and the dosage experiment. Blood samples were drawn after the procedure. Tissue samples were collected at the termination of the studies. Oxidative stress was followed upon the determination of malondialdehyde (MDA), reduced glutathione (GSH), thiol group (-SH), and superoxide dismutase (SOD) plasma levels. In addition the expression patterns of SOD-mRNA (messenger ribonucleotic acid) and PPARG-mRNA were measured with RNA extraction, complementary deoxyribonucleotic acid (cDNA) synthesis and semi-quantitative reverse transcription polymerase chain reaction (PCR) analysis. To evaluate the visible changes in muscle structure in the investigated groups histological investigations were performed as well.

In the ***third study*** we investigated the effects of phosphodiesterase inhibition by pentoxifyllin (PTX) on infrarenal aortic occlusion. Recently has been established that PTX could modulate the inflammatory response in ischemia reperfusion injury and sepsis. We hypothesized that single shot, increased dose of PTX treatment in conjunction with its known hemorheological effects decreases the extent of developing ischemia-reperfusion injury and can attenuate the local and systemic inflammatory response [85]. We investigated the developing ischemia-reperfusion by measuring MDA, GSH, -SH, SOD. TNF-alpha, IL-6

plasma levels were determined to follow and describe the inflammatory response after infrarenal aortic ischemia and reperfusion.

3. INHIBITION OF GLUTATHIONE S-TRANSFERASE BY ETHACRYNIC ACID AUGMENTS THE ISCHAEMIA-REPERFUSION DAMAGES AND APOPTOSIS AND ATTENUATES THE POSITIVE EFFECT OF ISCHAEMIC POSTCONDITIONING IN BILATERAL ACUT HINDLIMB ISCHAEMIA RAT MODEL

3.1. Introduction

In vascular surgery the acute limb ischemia is a severe, potentially life-threatening state. In this case it is necessary to perform a prompt revascularization. The duration of acute ischemia could also be serious, thus after the revascularization we always have to face reperfusion injury. The severity of reperfusion injury depends on the ischemic time, the mass of ischemic tissue and the collateral circulation of the affected tissue. In the surgical research it is of real clinical importance to reduce the extent of reperfusion injury associated pathways. [15, 16, 17]

Reperfusion injury is an inherent response to the restoration of the blood flow after ischemia, and is initiated at the early moments of reperfusion, lasting potentially for days. The severity of the oxidative stress and the generalized inflammatory response depends on the volume of the ischemic tissue, the ischemic time, the collateral circulation of the extremity and the general state and balance between the pro-oxidant agents and the endogenous antioxidant enzyme system. Under pathophysiological circumstances such as ischemia, the amount of reactive oxygen intermediates (ROI) and nascent oxygen free radicals is beyond the protecting capacity of the endogenous antioxidant system, thus, the oxidative stress will develop. [18, 19] The mechanisms of reperfusion-induced cell death are not totally understood, but it seems that the occurrence of oxidative stress related to the generation of ROS (reactive oxygen species) may play an important role. [20]

Glutathione S-transferases are members of the endogenous antioxidant enzyme system. GSTs represent three major families of proteins: cytosolic, mitochondrial, microsomal transferases, of which the cytosolic GSTs create the greatest family. [21] It has been recently

published that these enzymes catalyze the conjugation of reduced glutathione to electrophilic compounds, thus, that's the way they are involved in the detoxification of endogenous as well as exogenous substances. [22, 23] Several isoenzymes of GSTs play an important role in the regulation of pathways involved in cell survival and death signaling. In this non-enzymatic way, GSTs' role is to segregate the mitogen activated protein kinase in a complex, thus preventing it from acting on additional targets. [24]

Ethacrynic acid is a widely spread diuretic drug and in addition, a potent inhibitor of GST enzymes. Some pathways and mechanisms were discovered for the inhibition of the GST enzymes by EA. EA has been shown to be a substrate of reduced glutathione, on the other hand nonenzymatic GSH conjugation of EA also exist. Additionally it was shown that the EA glutathione conjugate was an inhibitor of GSTs as well due to its stronger affinity for the enzymes. So EA itself inhibits GSTs through reversible covalent binding and the EA glutathione conjugate is another strong inhibitor of GSTs. [25]

In the literature it has been recently published that short suspensions in the early reperfusion with brief ischemic episodes after a prolonged ischemic period may moderate the total IR (ischemia-reperfusion) injury. This procedure has been termed ischemic postconditioning. Postconditioning seems to be a simple-to-apply procedure in the interventional cardiology and potentially effective in reducing reperfusion injuries. [26]

To evaluate the effect of GST inhibition and ischemic postconditioning on ischemia-reperfusion damages in a lower extremity ischemia animal model, we monitored the serum malondialdehyde, reduced glutathione, thiol group levels, superoxide-dismutase activity, as well as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) concentrations and the expression of two proapoptotic signaling proteins, phospho-JNK (Jun N-terminal kinase) and phospho-p38 in the early reperfusion period. To confirm the effectivity of GST inhibition by EA we measured the active GST-alpha concentrations in the plasma. To demonstrate the structural changes in muscle fibers tissue samples of quadriceps femoris muscle group were taken.

3.2. Materials and methods

3.2.1. Animal model

60 male Wistar rats, weighed between 200-250 g were used in the present study from Charles River Breeding Laboratories (Hungary, Isaszeg). The animals were housed in individual cages in a temperature (25 ± 2˚C), light controlled (12 hours light-dark cycle) and air-filtered room with free access to food and water. Food was withdrawn 12 hours prior to experiment. The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

3.2.2. Aortic ischemia-reperfusion model

The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (500 mg / 10 ml) and diazepam (10 mg / 2 ml). The ratio was 1:1 (0.2 ml / 100 g = 5 mg ketamine + 0.5 mg diazepam / 100 g) and the animals were placed on a heated pad. ECG was placed and the carotid artery was catheterized (22 gauge) for blood pressure measurement (Siemens Sirecust 1260, Düsseldorf, Germany). The skin was disinfected and a midline laparotomy was performed. 2 ml of warm saline was injected into the abdominal cavity to help maintain the fluid balance. The inferior mesenteric vein was catheterized for collecting blood samples, fluid equilibration and supplemental anesthetic. The abdominal aorta was exposed by gently deflecting the intestine loops to the left. After fine isolation of the infrarenal segment, an atraumatic microvascular clamp was placed on the aorta for 60 minutes. The abdomen was then closed and the wound was covered with warm, wet compress to minimize heat and fluid losses. The microvascular clamp was then removed and the infrarenal abdominal aorta (IAA) was reperfused for 120 minutes. Aortic occlusion and reperfusion was confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta.

3.2.3. Administration of ethacrynic acid

We extrapolated the human diuretical dose of EA to rat. 86 mg of EA was suspended in 2 ml of 96% ethanol and 8 ml of saline solution and the concentration was 8.6 mg/ml. The solution contained approximately 20% etanol (EtOH). The dose of EA was 8.6 mg/kg and the solution was administered to the animals intraperitoneally 24 hours and 1 hour before the operation. To determine the effectivity of GST inhibition the biologically active alpha-GST concentration was measured in each group using Rat GST-alpha ELISA kit (Abnova, Taipei City, Taiwan), following the manufacturers protocol. This method determines the free i.e. biological active alpha-GST concentration.

3.2.4. Protocol of ischaemic postconditioning

Those groups wherein the animals underwent ischemic postconditioning, after the ischemic phase intermittent 15 seconds reperfusion – 15 seconds ischemic periods were applied four times.

3.2.5. Experimental groups

Rats were divided into six groups (10 rats in each group). In the control group a midline laparotomy was performed for three hours without following intervention. 20% EtOH vehicle the same volume with the EA-EtOH solution (1 ml/kg) was administered to the animals intraperitoneally 24 hours and 1 hour before the operation (control). The aorta in the second group was closed for 60 min and then a 120 min of reperfusion followed (IR). Rats in the third group underwent 60 minutes ischemia, after the ischemic phase postconditioning was performed followed by a 120 minutes reperfusion phase (PC). In the fourth, fifth and sixth groups the animals were treated with EA as well (EA-control, IR/EA, PC/EA). **(Fig. 1.)**

Peripheral blood samples and biopsy from quadriceps muscle were collected from the animals at the end of the reperfusion phase. The serum and tissue samples were harvested and stored at minus 78˚C until biochemical assays.

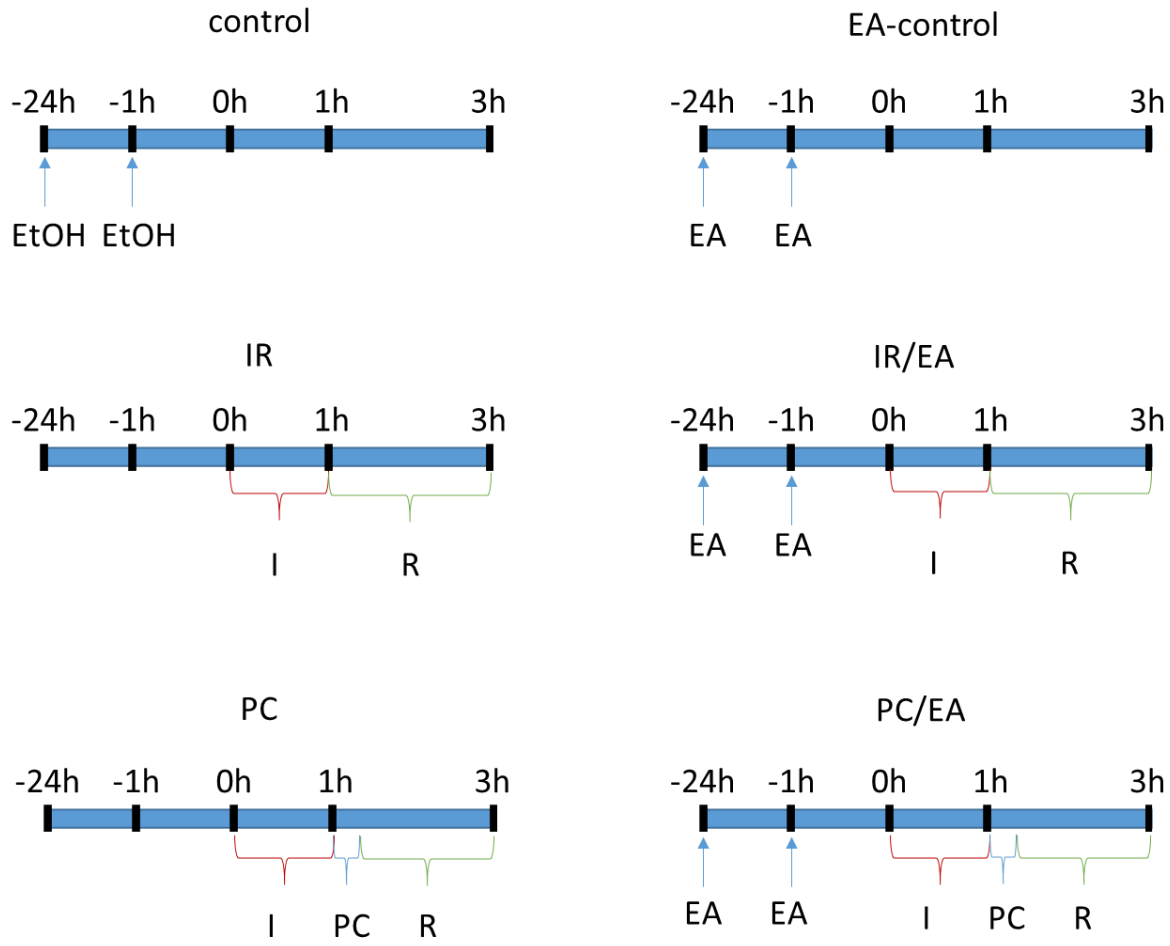


Fig.1. Investigation groups. EtOH: ethanol, EA: ethacrynic acid, I: ischemia, R: reperfusion, PC: postconditioning.

3.2.6. Analysis of oxidative stress parameters

Measurement of malondialdehyde: MDA is a marker for the quantification of lipid peroxidation in cell membranes. MDA was determined in anticoagulated whole blood, by photometric method of Placer, Cushman and Johnson. [27]

Measurement of reduced glutathione and plasma thiol-groups: Reduced glutathione is the predominant low-molecular-weight thiol in cells. Because of the cysteine residue GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances. GSH concentrations reduce markedly in response to protein malnutrition and oxidative stress. [28] GSH and plasma SH levels were determined in anticoagulated whole blood (ethylenediaminetetraacetic acid (EDTA)) by Ellman's reagent according to the method of Sedlak and Lindsay. [29]

For measuring of superoxide dismutase activity in serum we used Superoxide Dismutase Assay Kit (Trevigen Inc., Gaithersburg, USA), following the manufacturers protocol. This method determines the free i.e. biological active SOD activity.

3.2.7. Serum TNF-alpha and IL-6 quantification

For measuring TNF-alpha and IL-6 concentration in serum we used Rat TNF-alpha and Rat IL-6 ELISA kit (R&D Systems, Inc., Minneapolis, USA), following the manufacturers protocol. These methods determine the free i.e. biological active TNF-alpha and IL-6 concentrations.

3.2.8. Serum alpha-GST quantification

To determine the effectivity of GST inhibition the biologically active alpha-GST concentration was measured in each group using Rat GST-alpha ELISA kit (Abnova, Taipei City, Taiwan), following the manufacturers protocol. This method determines the free i.e. biological active alpha-GST concentration.

3.2.9. The western-blot analysis of proapoptotic (JNK, p38) signaling pathways

Fifty milligrams of quadriceps muscle samples were homogenized in ice-cold TRIS buffer (50 mM, pH 8.0), the homogenate was pelleted, and the supernatant was measured by

bicinchonicic acid reagent and equalized for 1 mg/ml protein content in Laemmli solution for Western blotting. The samples were harvested in 2X concentrated SDS-polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking (2 h with 3% nonfat milk in TRIS-buffered saline) membranes were probed overnight at 4°C with antibodies recognizing the following antigenes: phospho-SAPK/JNK (Thr^{183}/Tyr^{185}, 1:1000 dilution), pospho-p38 MAPK (Thr^{180}/Tyr^{182}, 1:1000 dilution), (Cell Signaling Technology, Danvers, MA, USA). Membranes were washed six times for 5 min in TRIS-buffered saline (pH 7.5) containing 0.2% Tween (TBST) before addition of goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3000 dilution; Bio- Rad, Budapest, Hungary). Membranes were washed six times for 5 min in TBST and the antibody-antigen complexes were visualized by means of enhanced chemiluminescence. The results of Western blots were quantified by means of Scion Image Beta 4.02 program. All experiments were repeated four times.

3.2.10. Histological examinations

The animals were terminated at the end of the experiment and biopsy was taken from quadriceps femoris muscle. The fragments of muscle did not contain well-identified fascia. The definite aim of the biopsy was to register the qualitative differences in changes between the animal groups, firstly the transformations in the striated muscular tissue. 5-6 paraffin-embedded blocks were made from striated muscle-pieces, and sample slices were prepared staining by hematoxylin and eosin.

The biopsies were made with the following method:

The fresh tissue was fixed in 10% neutral buffered formalin. Sample preparation was performed with a tissue processor equipment (Thermo Shandon Path centre, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sectioning was performed with a sledge microtome (5 µm, Reichert Optische Werke AG, Vienne, Austria) from the paraffin-embedded blocks, and staining was carried out with a carousel-type slide stainer (Thermo Varistain 24-4, Thermo Fisher Scientific Inc., Waltham, MA, USA) with hematoxylin and eosin at the Medical School University of Pécs, Department of Pathology, Pécs, Hungary. To evaluate the histological slices we used the Pannoramic Viewer software (3DHistec Ltd.) and 200x magnification.

3.2.11. Statistical analysis

All values are expressed as means ± SEM. Differences between the variances of the groups were assessed with one-way analysis of variance (ANOVA) and when the results were significant we used adequate post-hoc tests for multiple comparisons. For comparing the treated groups to the control group we performed in case of each investigated parameters Dunnett's test. We used Sidak post-hoc test for comparisons across multiple different groups. Multiple comparisons tests resulted in adjusted p-values, each p-value is adjusted to account for multiple comparisons. We performed five-five comparisons (Dunnett's and Sidak) per investigated parameter. T-tests were performed independently to show the differences between the investigated groups. Data were considered significant when p-value was less than 0.05.

3.3. Results

3.3.1. Plasma malondialdehyde levels

We measured in an in vivo animal model the values of malondialdehyde plasma-level indicating membrane damage and lipid peroxidation. The MDA concentration was significantly higher in four groups (IR, PC, IR/EA, PC/EA) comparing to the control group (72.6 ± 1.3; 68.9 ± 1.2; 78.7 ± 2.5; 74.1 ± 2.1 nmol/ml vs. 61.4 ± 2.4 nmol/ml / $p<0.0001$; $p= 0.017$; $p< 0.0001$; $p= 0.006$). We performed one-way ANOVA followed by Dunnett's test. Our data showed significant ($p=0.022$) differences between EA-control and IR/EA groups. MDA level was significantly higher in the IR/EA group (67.0 ± 2.5 nmol/ml vs. 78.7 ± 2.4 nmol/ml). We performed one way ANOVA followed by Sidak test. **(Fig.2.)**

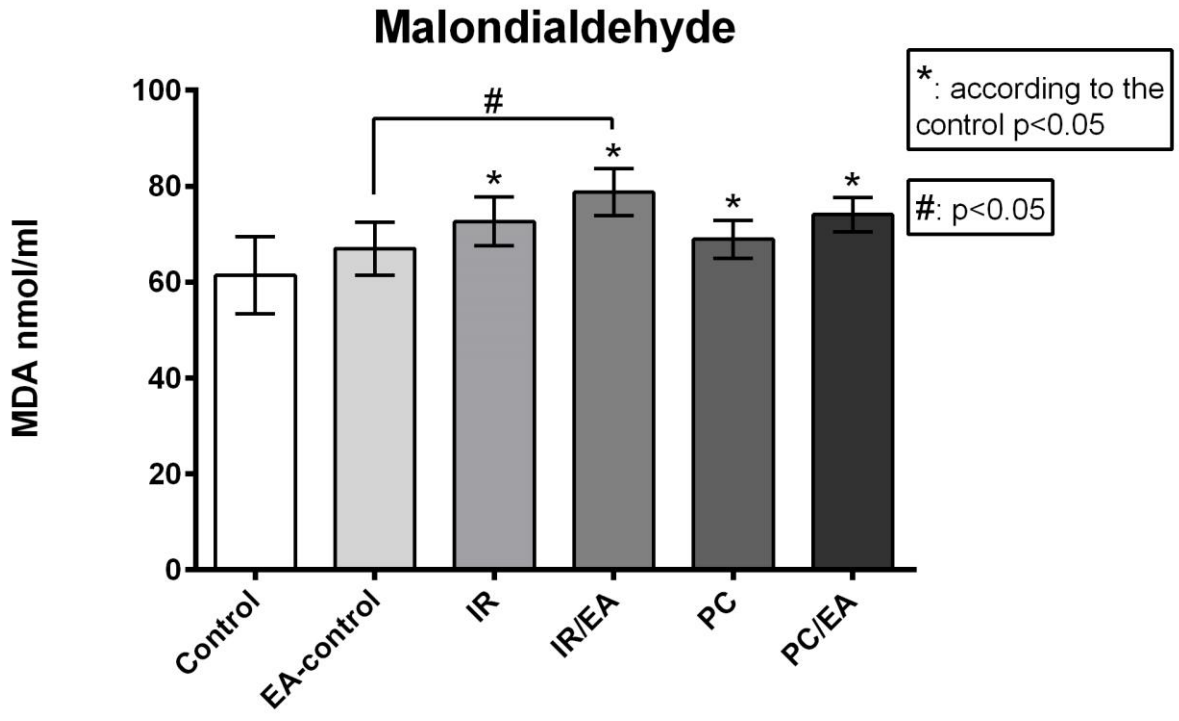


Fig. 2. Malondialdehyde concentrations in the experimental groups. MDA signs the severity of lipidperoxidation. *: $p<0.05$ vs. control; #: $p<0.05$ between the signed groups; error bars: SD

3.3.2. Reduced glutathione levels (GSH)

The values of reduced glutathione levels were significantly lower in four groups (IR, PC, IR/EA, PC/EA comparing to the control group (720.9 ± 15.7; 771.8 ± 18.6; 666.7 ± 16.9; 746.8 ± 15.8 nmol/ml vs. 876.2 ± 20.9 nmol/ml / p <0.0001; p =0.0006; p <0.0001; p =0.0016). We found in the IR/EA and PC/EA groups lower values than in similar groups without administration of EA but these data could not reach the level of significance (IR, PC) (666.7 ± 16.9 nmol/ml vs. 720.9 ± 15.7 nmol/ml and 746.8 ± 15.8 nmol/ml vs. 771.8 ± 18.6 nmol/ml / p =0.39; p = 0.95). In postconditioned (PC) group the values were significantly higher than in non-conditioned (IR) group (771.8 ± 61.8 nmol/ml vs. 720.9 ± 58.7 nmol/ml (p =0.047)), however this protecting factor of postconditioning between the similar groups in the presence of EA was not significant. **(Fig.3.)**

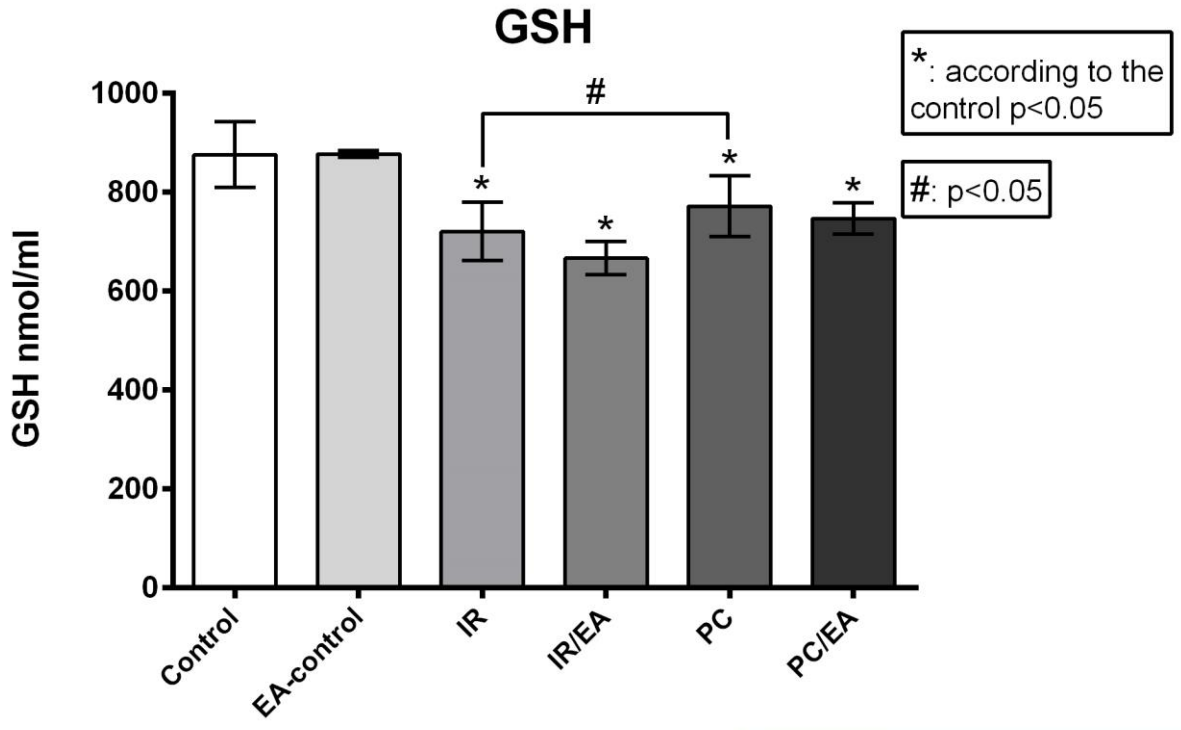


Fig. 3. Plasma concentrations of reduced glutathione in the investigated groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.3. Plasma thiol groups (-SH)

We detected in the IR/EA group significantly lower level of –SH comparing to control group. (37.5 ± 2.5 nmol/ml vs. 50.4 ± 2.7 nmol/ml / p =0.024) There was no significant difference in –SH level between other groups. **(Fig.4.)**

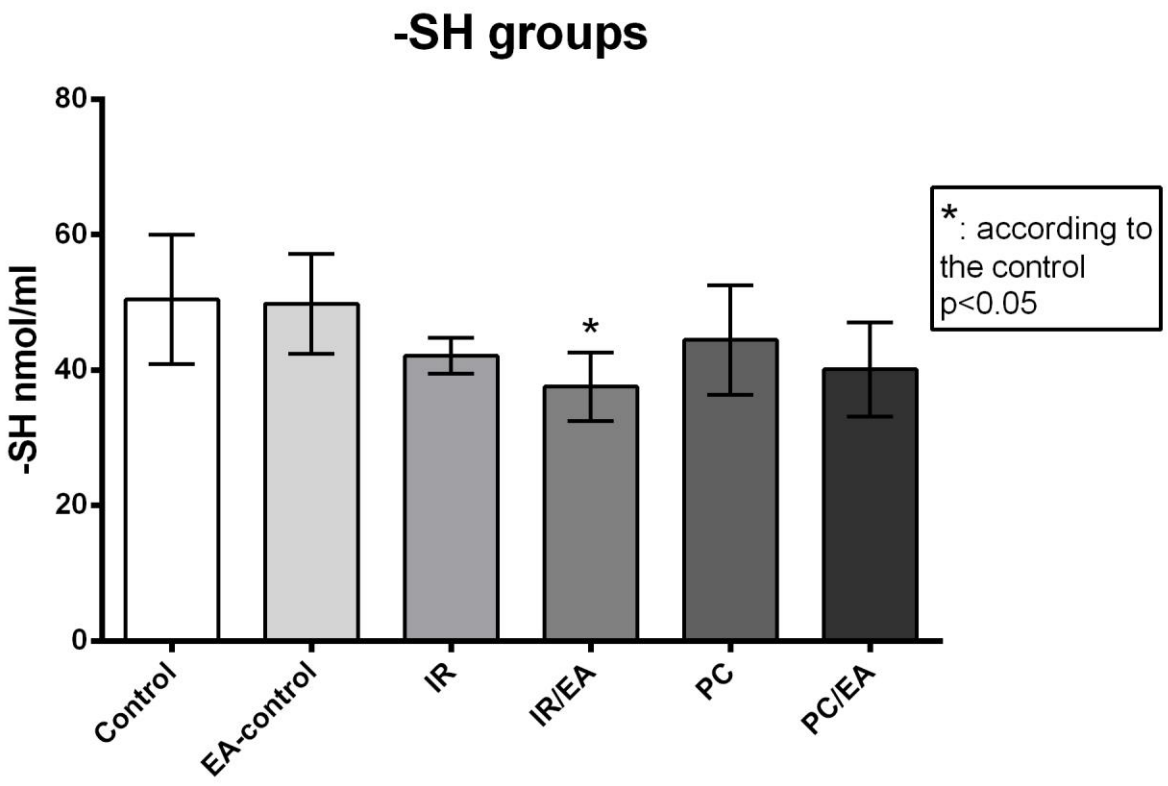


Fig. 4. Concentrations of –SH groups in the plasma. *: p<0.05 vs. control; error bars: SD

3.3.4. Enzyme activity of superoxide dismutase (SOD)

We have detected in three investigated groups significantly lower (IR, IR/EA, PC/EA) and in one group significantly elevated (PC) SOD activity comparing to the control group (578.6 ± 43.0; 475.2 ± 30.8; 495.1 ± 24.5; 1132.0 ± 69.3 U/l vs. 909.3 ± 30.1 U/l / p <0.0001; p <0.0001; p =0.001; p =0.003). In PC group we have found significantly higher values comparing to the IR group (1132.0 ± 69.3 U/l vs. 578.6 ± 43.0 U/l (p <0.0001). We have measured in the PC/EA group significantly decreased SOD activity comparing to the PC group (495.1 ± 24.5 U/l vs. 1132.0 ± 69.3 U/l (p <0.0001). **(Fig.5.)**

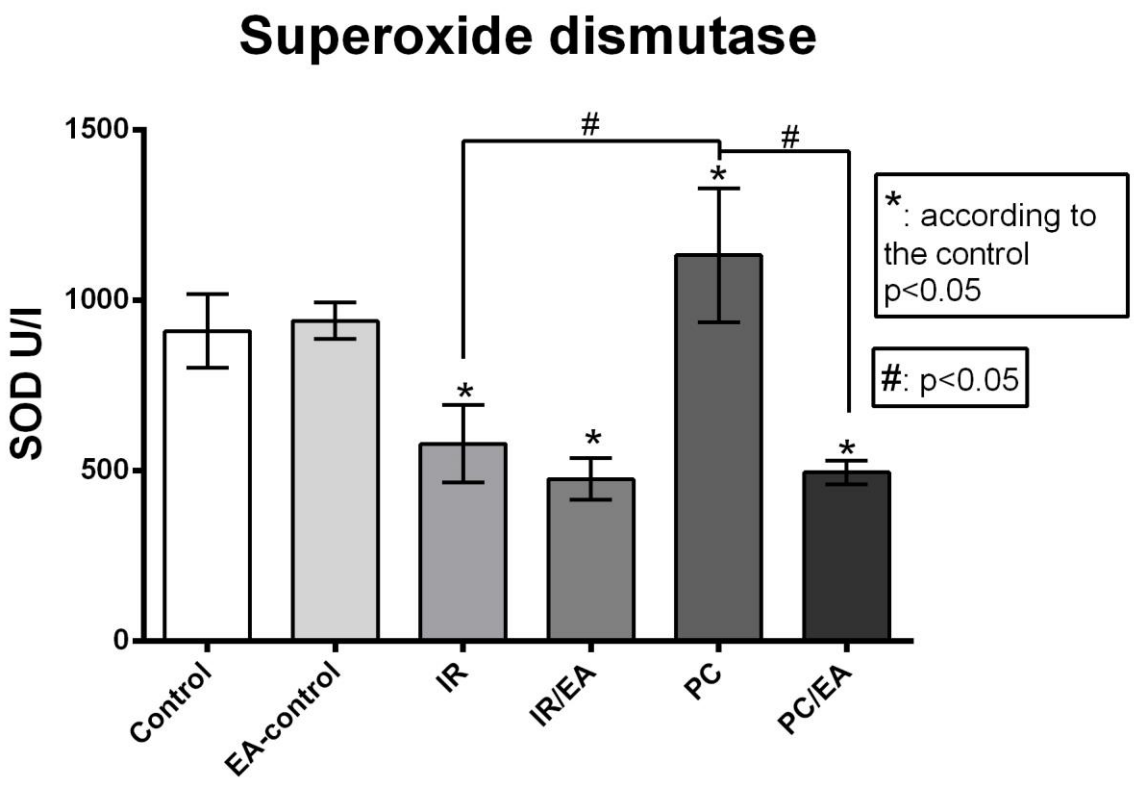


Fig. 5. Enzyme-activity of superoxide dismutase in the investigated groups.; *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.5. Serum TNF-α levels

In the study we measured the TNF-α levels in the groups. The values were significantly higher in three groups (IR, IR/EA, PC/EA) than in the control group (22.1 ± 0.4 pg/ml, 23.2 ± 0.9 pg/ml, 21.5 ± 0.6 pg/ml vs. 18.3 ± 0.3 pg/ml / p =0.0003; p <0.0001; p =0.011). In the postconditioned group we have not found significant elevation in the level of TNF-α comparing to the control group (20.7 ± 0.4 pg/ml vs. 18.3 ± 0.3 pg/ml / p =0.057). This anti-inflammatory protecting effect of postconditioning was not marked in the presence of EA (PC/EA vs. control; 21.5 ± 0.6 pg/ml vs. 18.3 ± 0.3 pg/ml / p =0.011). **(Fig.6.)**

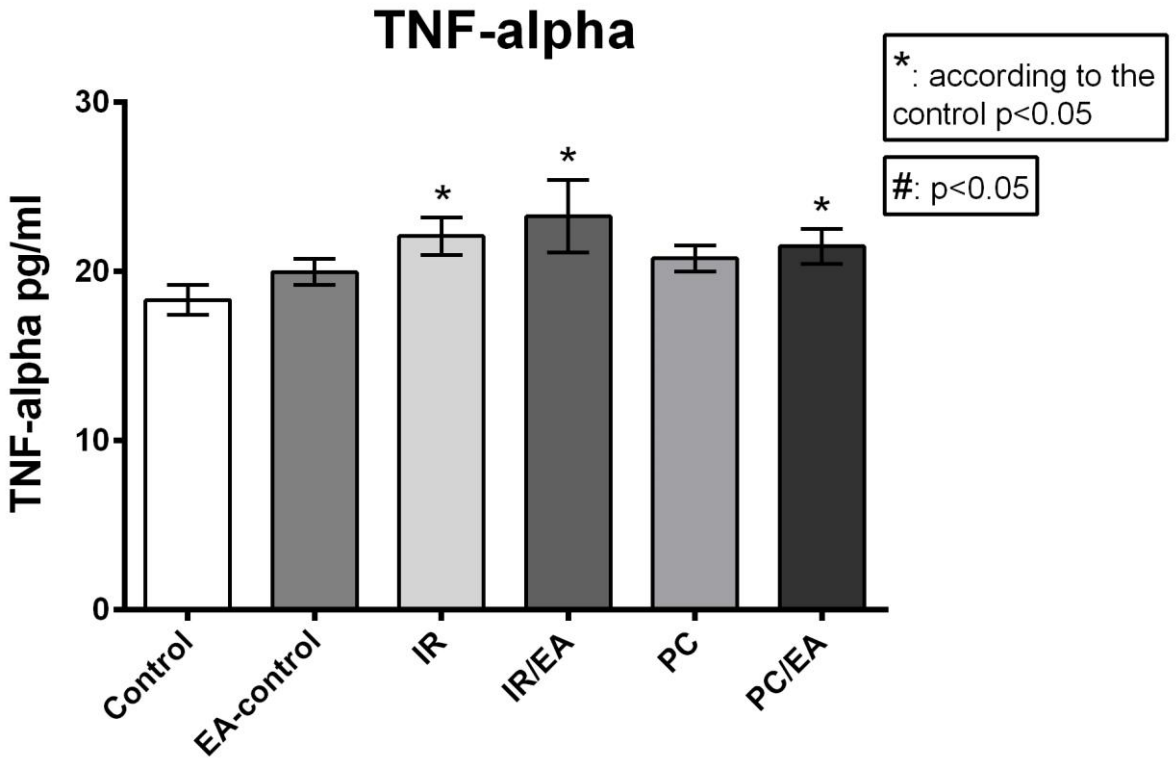


Fig. 6. TNF-alpha concentrations shows the grade of inflammatory response in the groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.6. Serum interleukin-6 (IL-6)

We investigated the serum IL-6 levels in our groups. The values were significantly higher in three groups (IR, IR/EA, PC/EA) than in the control group (139.9 ± 4.7; 223.0 ± 10.0; 209.4 ± 3.8 pg/ml vs. 108.3 ± 3.2 pg/ml / p =0.002; p <0.0001; p< 0.0001). IR in the presence of EA caused a significantly elevated level of IL-6 comparing to the IR group without EA administration (IR/EA vs. IR; 223.0 ± 10.0 pg/ml vs. 139.9 ± 4.7 pg/ml (p<0.0001)). Postconditioning decreased significantly the IL-6 level without EA (IR vs. PC; 139.9 ± 4.7 pg/ml vs. 111.3 ± 0.8 pg/ml (p =0.027)). Postconditioning did not temper significantly that elevation of IL-6 level if the animals were co-treated with IR and EA administration (IR/EA vs. PC/EA; 223.0 ± 10.0 pg/ml vs. 209.4 ± 3.8 pg/ml (p =0.66)). **(Fig.7.)**

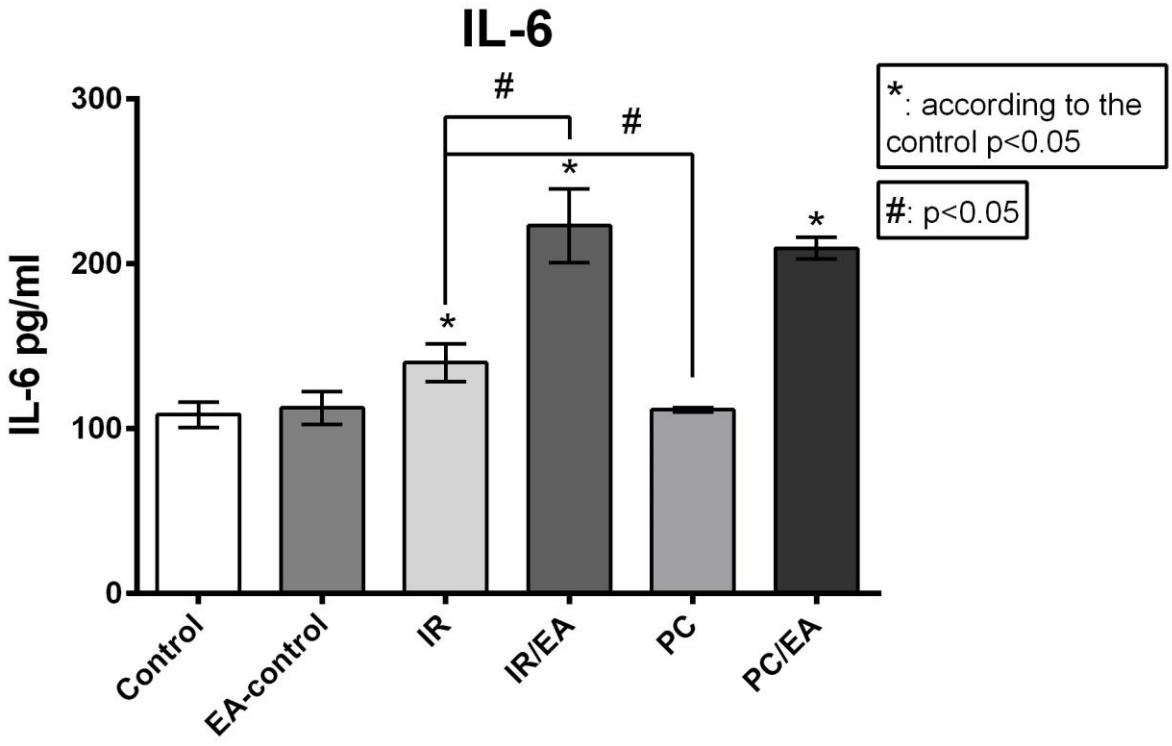


Fig. 7. IL-6 plasma-concentrations shows the grade of inflammatoric response in the groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.7. Serum alpha-GST

To evaluate the effectivity of GST inhibition by ethacrynic acid we measured the serum alpha-GST concentrations in the investigated groups. These concentrations stem from the biologically active enzymes and correlate with the enzyme activity (Abnova). The values were significantly lower in the EA administered groups (EA, IR/EA, PC/EA)) according to the control group (17.5 ± 1.95 ng/ml, 18.8 ± 2.2 ng/ml, 17.9 ± 1.1 ng/ml vs. 54.2 ± 2.5 ng/ml / p <0.0001 in all three groups). We have found significantly elevated alpha-GST concentration in the IR group according to the control and PC group (IR vs. control; 85.3 ± 2.3 ng/ml vs. 54.2 ± 2.5 ng/ml; IR vs. PC; 85.3 ± 2.3 ng/ml vs. 65.1 ± 2.5 ng/ml / p <0.0001 in all two groups). **(Fig.8.)**

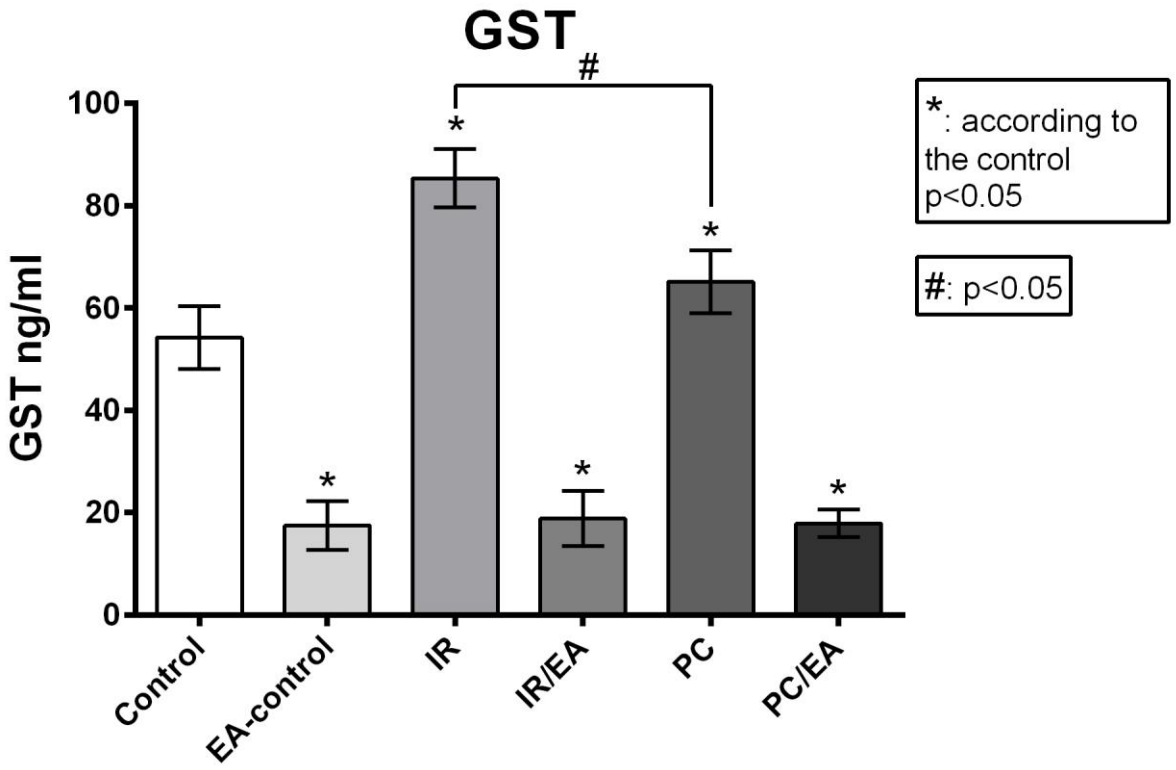


Fig.8. Alpha-GST concentrations show the grade of the GSTs' inhibition by EA in the groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.8. Western blot of proapoptotic signaling pathways (p38, JNK)

To characterize the expression and phosphorylation of two proapoptotic signaling proteins (phospho-JNK, phospho-p38) we used Western blot analysis to separate and establish them. We found that the expression of phospho-JNK and phospho-p38 was appreciably higher in the EA administered groups comparing to the control, IR and PC groups. **(Fig.9-10.)**

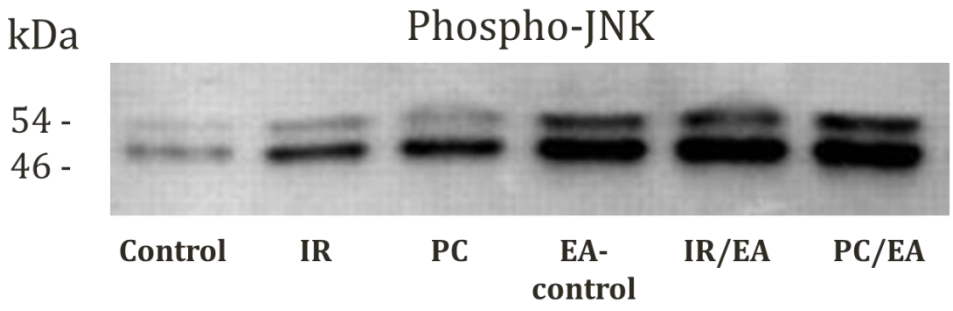


Fig. 9. Western blot analysis shows the phosphorylation and activation of proapoptotic signaling protein JNK.

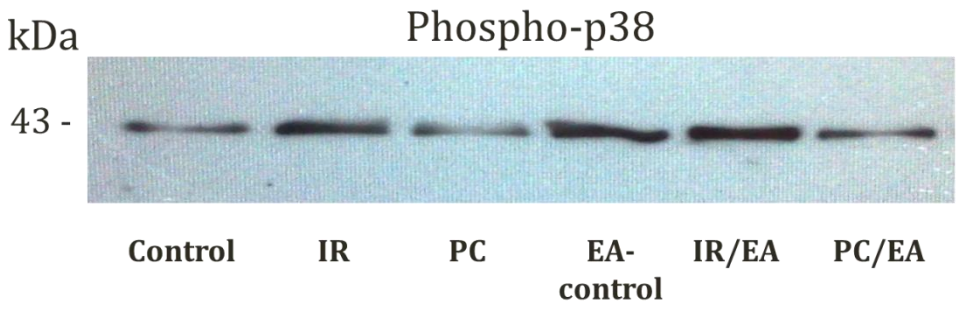


Fig. 10. Western blot analysis shows the phosphorylation and activation of proapoptotic signaling protein p38.

3.3.9. Histological results

(Fig.11.)

In the control group of animals the basic tissue structure is mainly kept in the striated muscle tissue, there is no fibrosis and necrosis cannot be defined with absolute certainty and neither significant inflammation cannot be observed. **(C)**

In the IR group the muscle fibers are swelled, irregular-shaped and the interstitial space between the fibers is pressed, decreased. Focal atrophy and necrosis were seen in the picture as well. **(IR)**

In the PC group the basic muscle structure is mainly kept. Muscle fibers are gently swelled but interstitial edema or necrosis cannot be defined. **(PC)**

In the slice of the EA-control group the muscle structure is undamaged, healthy, there is no necrosis or atrophy in the fibers. **(EA-C)**

We have seen the most severe damage of the muscle structure in the IR/EA group. The muscle fibers are disorganized, irregular-shaped and due to the edema the interstitial space between the fibers is increased. **(IR/EA)**

In the PC/EA group the damage of the muscle structure is severe. The muscle fibers are swelled, focal atrophy and necrosis can be seen in the picture as well. **(PC/EA)**

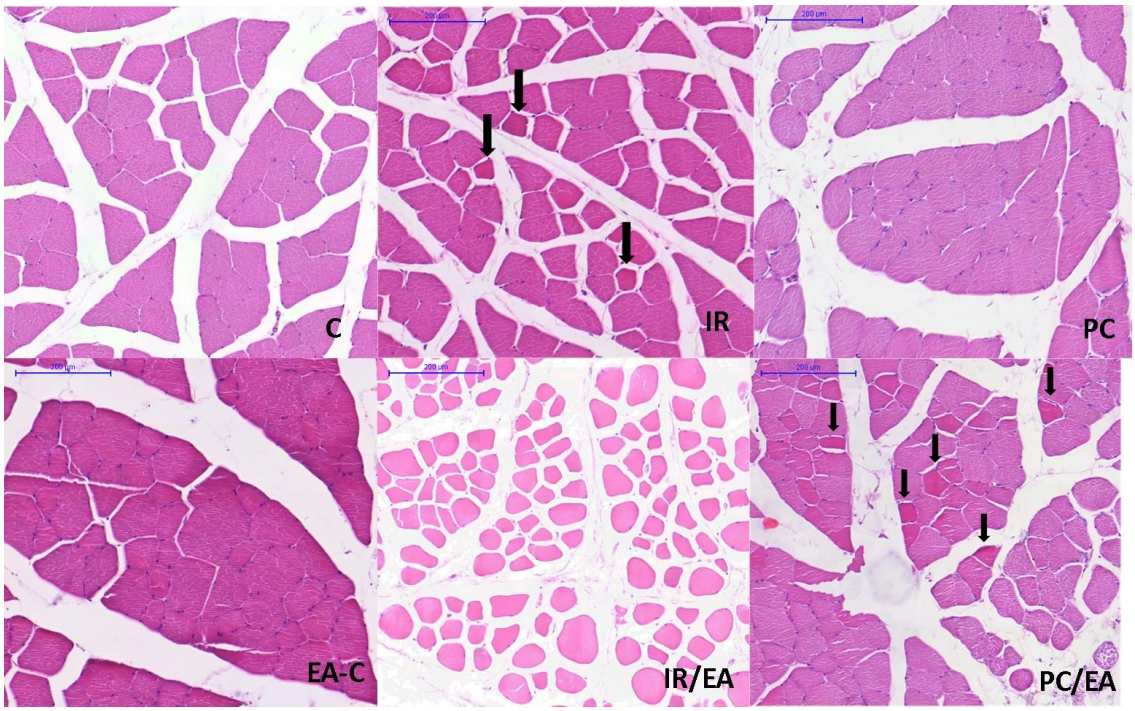


Fig.11. Quadriceps muscle slice, HE, 200x.

3.4. Discussion

Revascularization procedures performed on ischemized extremities will lead to reperfusion injury which is an integrated response to the restoration of blood flow after ischemia. Numerous factors could modulate the extent of oxidative stress and generalized inflammatory response. The effect depends on the duration of ischemia, the ischemic tissue volume and the general metabolic state of the organism (diabetes, drugs and chronic ischemia).

Ischemia will lead to decreasing level of intracellular adenosine triphosphate (ATP) and consecutive elevation of hypoxanthine. In the very early moments of reperfusion the oxygen appears in the cell and the xanthine oxidase catalyzed hypoxanthine-xanthine conversion will produce a mass of superoxide radicals. Through lipid peroxidation the superoxide radicals and other ROI will damage the membrane lipids, proteins and DNA. The endogenous antioxidant system tries to defend the cells and macromolecules against these injuries. [4] We have found that the simulated IR cause increased oxidative stress parameters which was further increased by administration of EA. The positive effect of ischemic postconditioning have been detected through the investigation of oxidative parameters, but this positive effect has not been seen in the presence of EA. We have found that –SH groups and TNF-alpha did not showed significant changes by EA. It can be explicable with the short time of IR. On the other way IL-6 showed significant changes due to injury of endothelial layer. This could be the first effect of the inflammatory response in reperfusion injury. [30]

ROI induce cytokine expression and leukocyte activation and will lead to local and systemic inflammation. Previous studies showed that the concentration of the proinflammatory cytokines (TNF-α, IL-6), produced by both macrophages and neutrophils, is elevated in the ischemic kidney and may have a pathophysiological role following IR injury. [31] TNF-α induces three pathways: apoptotic cell death; activation of MAPK pathway; and NF*K*B-pathway. [32] Through oxidative and inflammatory responses will develop a complex reperfusion injury.

Postconditioning is defined as short series of repetitive cycles of brief reperfusion and reocclusion of blood flow in the early moments of reperfusion. The phenomenon was

published for the first time by Vinten-Johansen et al. in myocardium and induced an effective cardioprotection. [12]

A main determinant of the cellular response to oxidative stress relates to the level and molecular form of glutathione. [33] A key factor that determines the level of glutathione is its utilization via glutathione S-transferase. [34, 35, 9] GSTs function through the conjugation of GSH and catalyzation attack on foreign compound or ROI will lead to generally forming less reactive materials that can be readily excreted. The capability of GST to alter level of cellular glutathione in response to production of ROI has been implicated in protection of cells from ROI-inducing agents. [36, 37]

The present study showed that GST inhibition could aggravate the IR injuries and strikingly attenuate the protecting effect of ischemic postconditioning and resulted in increasing phosphorylation of proapoptotic proteins. GST inhibition was associated with different activation of mitogen activated protein kinases. The GSTs have a quite wide polymorphism. The change in the polymorphic alleles could influence the individual ischemia-tolerance. There are three alleles of the GSTπ, A, B, C. Patients with “A” allele seem to be have reduced ischemic tolerance. This fact may play important role in reduction of postconditioning’s positive effect. [38] Earlier Balatonyi et al. have been investigated on cultured cardiomyocytes this reducing effect of GST inhibition on postconditioning. [30]

In our study we used EA for pharmacological inhibition of GST. EA has been shown to be a substrate of many of GST isozymes. [11]

In our experiment administration of EA resulted in marked increase of oxidative stress parameters, extended expression and phosphorylation of proapoptotic proteins, especially when animals were co-treated with IR. While ischemic postconditioning could decrease the concentrations of oxidative stress parameters in IR group, this positive effect could not be established in GST inhibited group co-treated with IR. The increased levels of ROI may explain the increased amount of phosphorylated proapoptotic proteins in GST inhibited group during IR and ischemic postconditioning. [39]

GSTs are associated with members of the MAPK pathways. These pathways play a role in the cell survival and death signaling. [40] GSTπ was described as the first inhibitor of JNK by direct protein-protein interaction. [17] JNK is a proapoptotic MAPK that plays a crucial role in cytotoxicity during numerous conditions including oxidative stress, nitrosative stress and

involved in stress response, inflammation, apoptosis and cellular proliferation. [41, 42] In normal conditions low JNK catalytic activity is maintained in the cell, GSTπ, JNK and c-Jun constitute a protein complex. [23] Under conditions of oxidative stress, the GSTπ-JNK complex dissociates, so that JNK regain its phosphorylating ability and is free to act on downstream gene targets resulting in induction of apoptosis. [43] We found that inhibition of GST by EA increases JNK activation by itself and diminishes the protective effect of postconditioning. This could be explained by the elimination of sequestration of JNK within a protein complex with GST. Furthermore, effective inhibition of GST may cause JNK activation as a result of oxidative stress due to hampered elimination of developing oxidants. Sun et al. have reported that attenuation in superoxide anion generation by postconditioning after hypoxia and reoxygenization was able to decrease the activation of JNK and p38 MAPKs, suggesting that modulation of MAPK signaling pathways are involved in the postconditioning-induced protection. [44] Recently, Balatonyi et al. have reported on cultured cardiomyocytes that inhibition of GST by EA augments the apoptosis as a result of simulated IR, furthermore abolishes the protective effect of ischemic postconditioning and this is presumably mediated by JNK, p38, ERK/p42-44 signaling pathways because the activities of these kinases change on this way during ischemic postconditioning. [45]

We have found that IR treatment caused a gently induction of p38 activation in rats quadriceps muscle which was further increased by administration of EA while the protective effect (the decreased phosphorylation of p38) of postconditioning could not develop.

3.5. Critique of the study

In our experiment we have investigated the oxidative stress parameters and inflammatory parameters. To evaluate the apoptosis we performed western blot test. In addition histological examination of the quadriceps muscle was performed. However, no functional data (force generation, flexion of the limb), extent of edema formation or clinical relevant data (compartment syndrome, renal failure) were assessed. Due to our reassuring results we have to continue the investigation series and expand to the clinical practice.

3.6. Conclusion

Our investigation results showed that the postconditioning, which is an endogenous adaptation mechanism, reduced the oxidative stress parameters and the inflammation with regard to the IR injury. The present study showed that inhibition of GST by EA leads to increased phosphorylation of JNK and p38 proapoptotic signaling proteins and abolishes the protective effect of postconditioning. The clinical importance of this study is that postconditioning seems to be an effective, quick and simple method to decrease the reperfusion damages after surgery. In vascular surgery, the administration of EA to the patients could worsen the reperfusion injuries and the postconditioning could not ameliorate these worsening effects. So in case of patients suffering from arterial occlusive vascular disease, the replacement of this diuretic drug by another agent is worthy of considering.

4. EFFECTS OF A PPARG AGONIST ON ISCHEMIA-REPERFUSION INJURY IN BILATERAL HINDLIMB ISCHAEMIA RAT MODEL

4.1. Introduction

During revascularization surgery on patients suffering from peripheral vascular disease the affected limb will be temporarily excluded from the circulation. Following reconstruction of the vascular bed tissues of the region, which has been ischemic so far, will be overloaded by an increased volume and elevated blood pressure. This overload depending on the time of exclusion, location of occlusion and general tissue conditions (diabetes mellitus, endothelial dysfunction etc.) will result in reperfusion injuries. These clinical events are hard to be monitored; however they substantially influence the postoperative outcome of the disease. On the basis of this evidence, investigations aiming at the reduction of reperfusion injury represent crucial contribution to research in experimental surgery.

The peroxisome proliferator-activated receptor-γ (PPARG) is a member of the nuclear receptor superfamily. PPARs are ligand-dependent transcription factors that bind to specific peroxisome proliferators response elements at the enhancer sites of regulated genes. [46] They are implicated in adipocyte differentiation, insulin sensitivity and inflammatory processes [47, 48] and also down-regulate proinflammatory mediators in macrophages, mainly by inhibiting transcription of NF-kB-dependent inflammatory genes. [49, 50, 51] Synthesized PPARG agonists (PPARGA) are used as oral antihyperglycemic drugs in treating noninsulin-dependent diabetes mellitus. [52] Troglitazone was the first such drug approved for treating type 2 diabetes, but was withdrawn from the market in 1999 due to hepatic toxicity. [53] The effects of PPARG agonists were originally thought to be limited to controlling lipid metabolism and homeostasis. However, emerging evidence indicates that PPARG activation can regulate inflammatory responses, included inflammatory disorders of the central nervous system, inhibiting expression of a variety of pro-inflammatory molecules by a mechanism termed receptor-dependent transrepression. [54] Furthermore, beneficial effects of PPARG agonists on ischemia reperfusion injury have been previously documented in the intestine [55, 56, 57], lung [58], heart [59, 60, 61], kidney [62] and, more recently, also in the brain [63, 64].

4.2. Materials and methods

4.2.1. Animal care

Wistar albino rats (250-280 g) were housed two per cage in an air-filtered and light controlled (12:12 hour, light-dark cycle) room at 21±2 °C. Purina™ rat chow pellets and water were provided ad libitum.

4.2.2. Time course experiment

The rats were divided randomly into five groups. The first group was a group of operated rats that underwent ischemia followed by an hour-long reperfusion without being treated with PPARGA (operated untreated control). The other four groups included operated rats that underwent ischemia followed by an hour-long reperfusion with a PPARGA treatment (intravenously; final concentration of 100 µM) either 0 minute, 20 minutes, 40 minutes, or 60 minutes before reperfusion. Upon completion of the one hour reperfusion, the rats were anesthetized with intraperitoneal injection of ketamine (40 mg/kg) and diazepam (5 mg/kg) and evaluated. **(Fig.12.)**

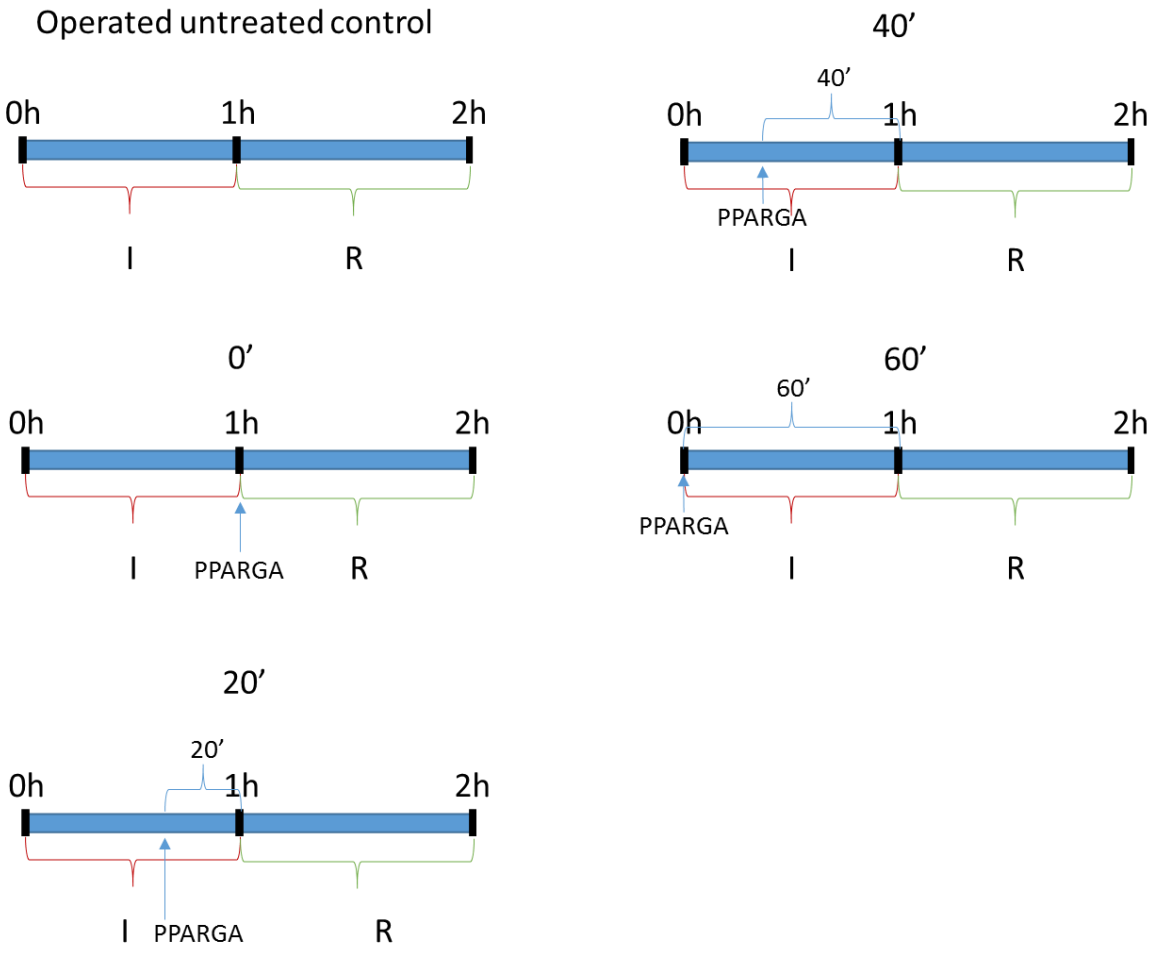


Fig.12. Investigation groups: I: ischemia, R: reperfusion, PPARGA: 100 µM.

4.2.3. Dosage experiments

Rats were randomly divided into five groups. The first group was a group of operated rats that underwent ischemia followed by an hour-long reperfusion without being treated with PPARGA (operated untreated control). The other four groups included operated rats that underwent ischemia followed by an hour-long reperfusion with an intravenous PPARGA treatment 20 minutes before reperfusion at a final concentration of either 10 µM, 50 µM, 100 µM, or 500 µM of PPARGA. **(Fig.13.)** Upon completion of the one-hour reperfusion, the rats were anesthetized with intraperitoneal injection of ketamine (40 mg/kg) and diazepam (5 mg/kg) and evaluated.

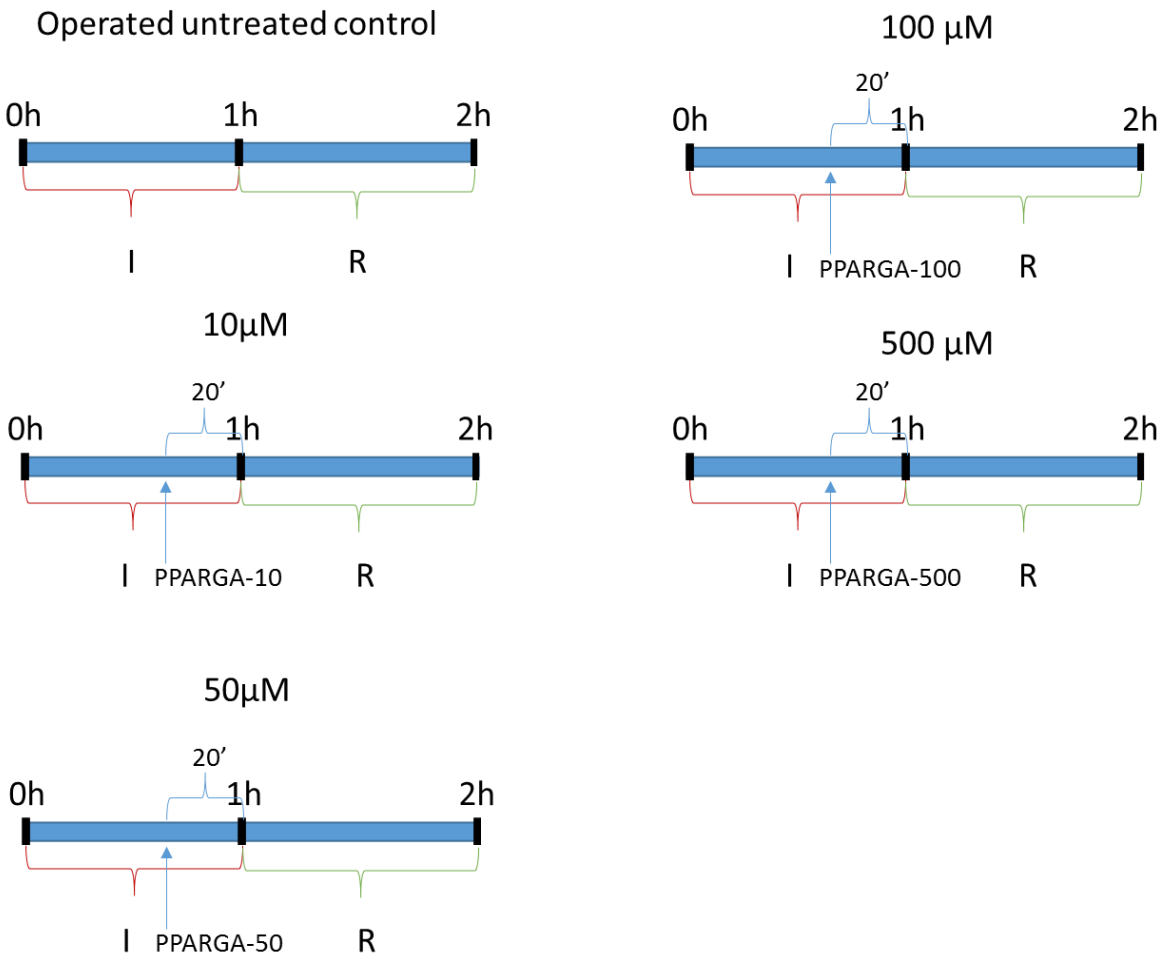


Fig.13. Investigation groups – dosage experiment I. I: ischemia, R: reperfusion.

In another dosage experiment, rats were randomly divided into five groups. The first group was a group of operated rats that underwent ischemia followed by an hour-long reperfusion without being treated with PPARGA (operated untreated control). The other four groups included operated rats that underwent ischemia followed by an hour-long reperfusion with an intravenous PPARGA treatment 40 minutes before reperfusion at a final concentration of either 10 µM, 50 µM, 100 µM, or 500 µM of PPARGA. **(Fig.14.)** Upon completion of the one-hour reperfusion, the rats were anesthetized with intraperitoneal injection of ketamine (40 mg/kg) and diazepam (5 mg/kg) and evaluated.

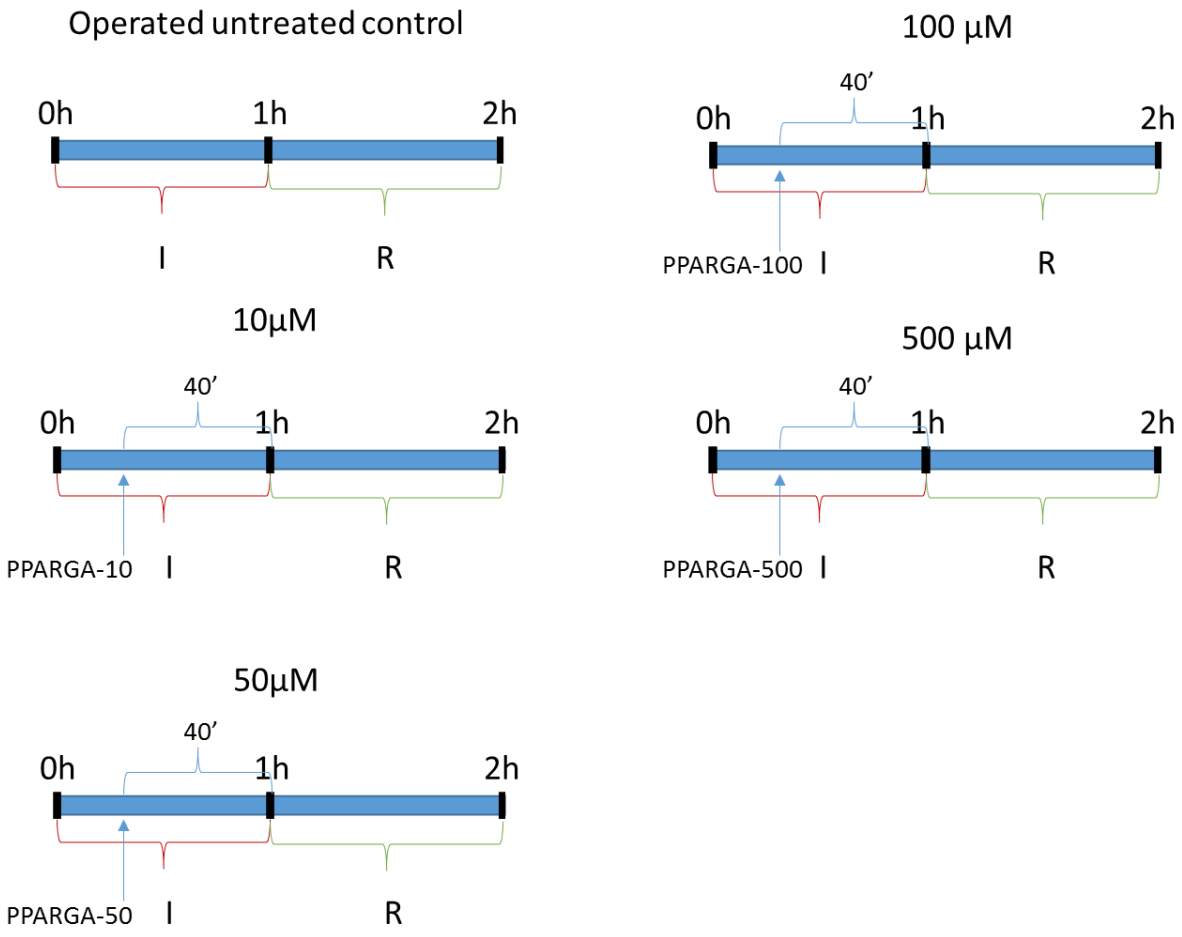


Fig.14. Investigation groups. I: ischemia, R: reperfusion.

4.2.4. Occlusion of infrarenal aorta

Following the anesthesia, an abdominal opening technique was performed by a midline laparotomy incision. **(Figures 15 A-F)** After repositioning the intestines, the aorta came into sight and was carefully isolated. After the isolation process, the aorta was occluded for one hour (ischemic period). The clamp was released at the end of the ischemic period to start the reperfusion for one hour. Right after the one-hour reperfusion treatment period, peripheral blood samples were collected and evaluated.

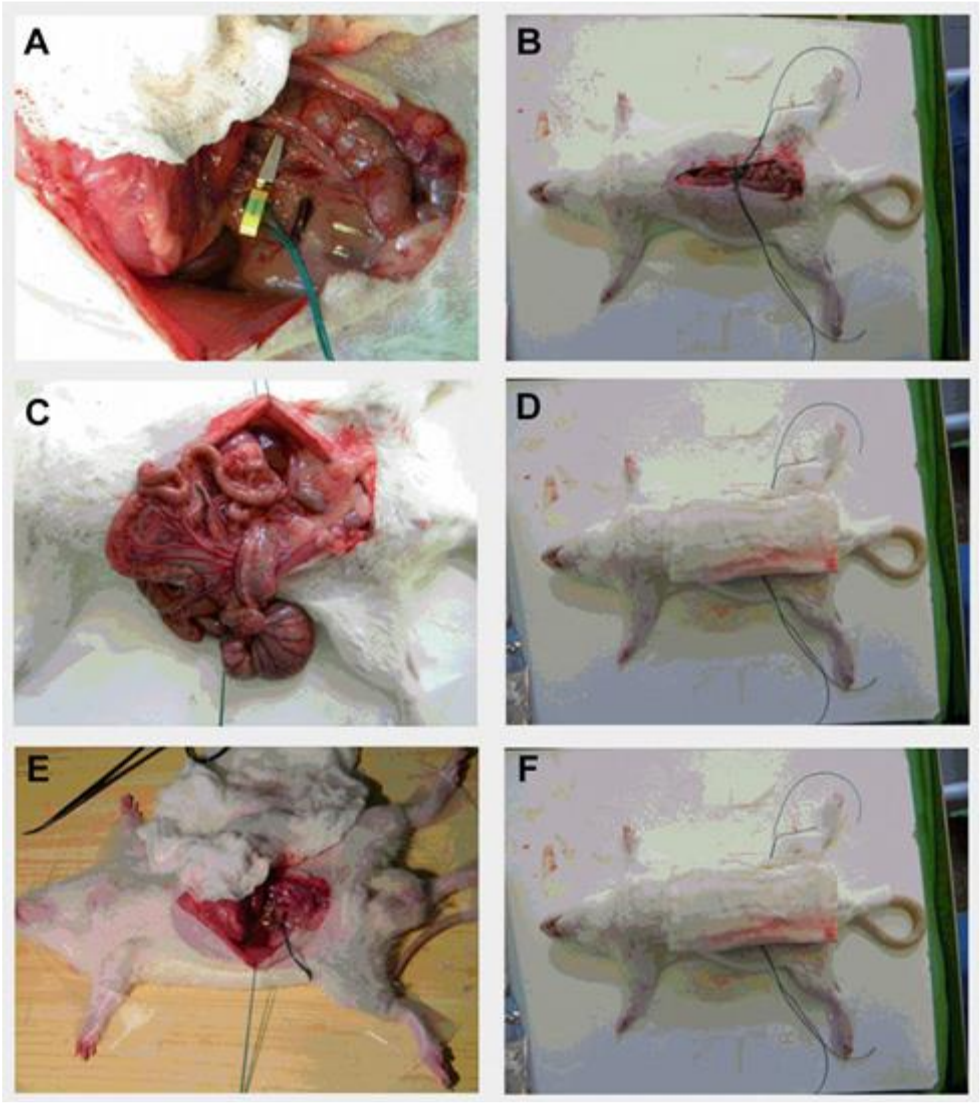


Figure 15. A-F are photographs of an operational method for inducing ischemia and performing reperfusion in rats. Figure 1A is a photograph of aorta occlusion. Figure 1B is a photograph taken during the ischemic period. Figure 1C is a photograph taken during the injection of PPARGA into the mesenteric vein during ischemia. Figure 1D is a photograph taken during the remaining time of the ischemic period. Figure 1E is a photograph taken during release of the clamp. Figure 1F is photograph taken during reperfusion.

4.2.5. PPARGA administration

A PPARGA solution was injected in an appropriate concentration (e.g., 10, 50, 100, or 500 µM at a final concentration) into the superior mesenteric vein at different time points (e.g., 20, 40, or 60 minutes before reperfusion of the ischemic period).

4.2.6. Measurement of oxidative stress markers

MDA levels were determined in anti-coagulated whole blood using a colorimetric assay as described elsewhere [32]. GSH levels, as opposed to oxidized GSH, were measured in anti-coagulated whole blood using a colorimetric assay as described elsewhere [33, 34]. SOD enzyme activity was measured in washed red blood cells using a colorimetric assay as described elsewhere [34]. The levels of total thiol groups (proteinbound and free sulfhydryl groups) were measured in anti-coagulated whole blood using a colorimetric assay as described elsewhere [33, 34].

4.2.7. RNA extraction

Total RNA was extracted from the renal tissue samples with TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions.

4.2.8. cDNA synthesis

Complementary DNA was synthesized from 5 µg DNase treated total RNA in 20 µL final volume using oligo(dT) primer and the M-MuLV reverse transcriptase from Fermentas RevertAid™ Reverse Transcriptase Kit according to the manufacturer's recommendations. The RTase was then inactivated at 70°C for 10-10 minutes.

4.2.9. Semi-quantitative reverse transcription PCR analysis

Two microliters of synthesized and 10-times diluted cDNA samples were used for PCR amplification by 1 units of Taq DNA polymerase (Fermentas, DreamTaq™ Green DNA Polymerase) in a total volume of 20 µL under the following conditions: 95°C for 5 min, followed by 30 cycles consisting of 30 seconds at 94°C then 30 seconds at 60°C (annealing

temperature of PPARG and SOD GAPDH primer pairs), 1 minute at 72°C. The procedure was ended at 72°C for 5 minutes. The number and temperature of cycles were optimized for each specific primer pair (Integrated DNA Technologies Inc.). Fifteen microliters of the PCR products were loaded and separated on 1.2% agarose gel containing ethidium bromide for visualization and photography experiments.

4.2.10. Histological analysis

Wistar albino rats (250-280 g) were sham operated (control without ischemia reperfusion treatment), subjected to ischemia followed by reperfusion (ischemia reperfusion control), or subjected to ischemia followed by reperfusion with PPARGA being injected intravenously at a final concentration of 100 µM at the 20th minute of the one hour long ischemic period, which was followed by a one-hour reperfusion. After the one-hour reperfusion periods, tissue samples were obtained from the hind limb (m. tensor fasciae latae) of the rats and fixed in 10% buffered formaldehyde prior to being processed in a paraffin-embedded block. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin.

4.3. Results

4.3.1. Results of the time course experiment

PPARGA was injected into the superior mesenteric vein at a final concentration of 100 µM at different time points (0, 20, 40, and 60 minutes) before reperfusion. The PPARGA treatment efficiently diminished the level of MDA at all time-points tested (Figure 12). Since MDA levels are a reliable oxidative stress marker, the decline of MDA levels indicates that PPARGA is a potent inhibitor of oxidative stress responses during the ischemia-reperfusion process. This ability of PPARGA to inhibit oxidative stress responses during the ischemia-reperfusion process was further supported by the observed increases in reduced GSH levels when PPARGA was administered 40 or 60 minutes prior to reperfusion and the observed increases in SOD activity at all timepoints tested **(Figure 16)**. Taken together, these results demonstrate that PPARGA, a compound reported to be an anti-inflammatory agent [65, 66, 67] inhibits oxidative stress during the ischemia-reperfusion process and can be used to reduce the injury caused by reperfusion following ischemia. These results also demonstrate that PPARGA is effective when administered at the time of reperfusion or when administered before reperfusion (e.g., 20, 40, or 60 minutes before reperfusion).

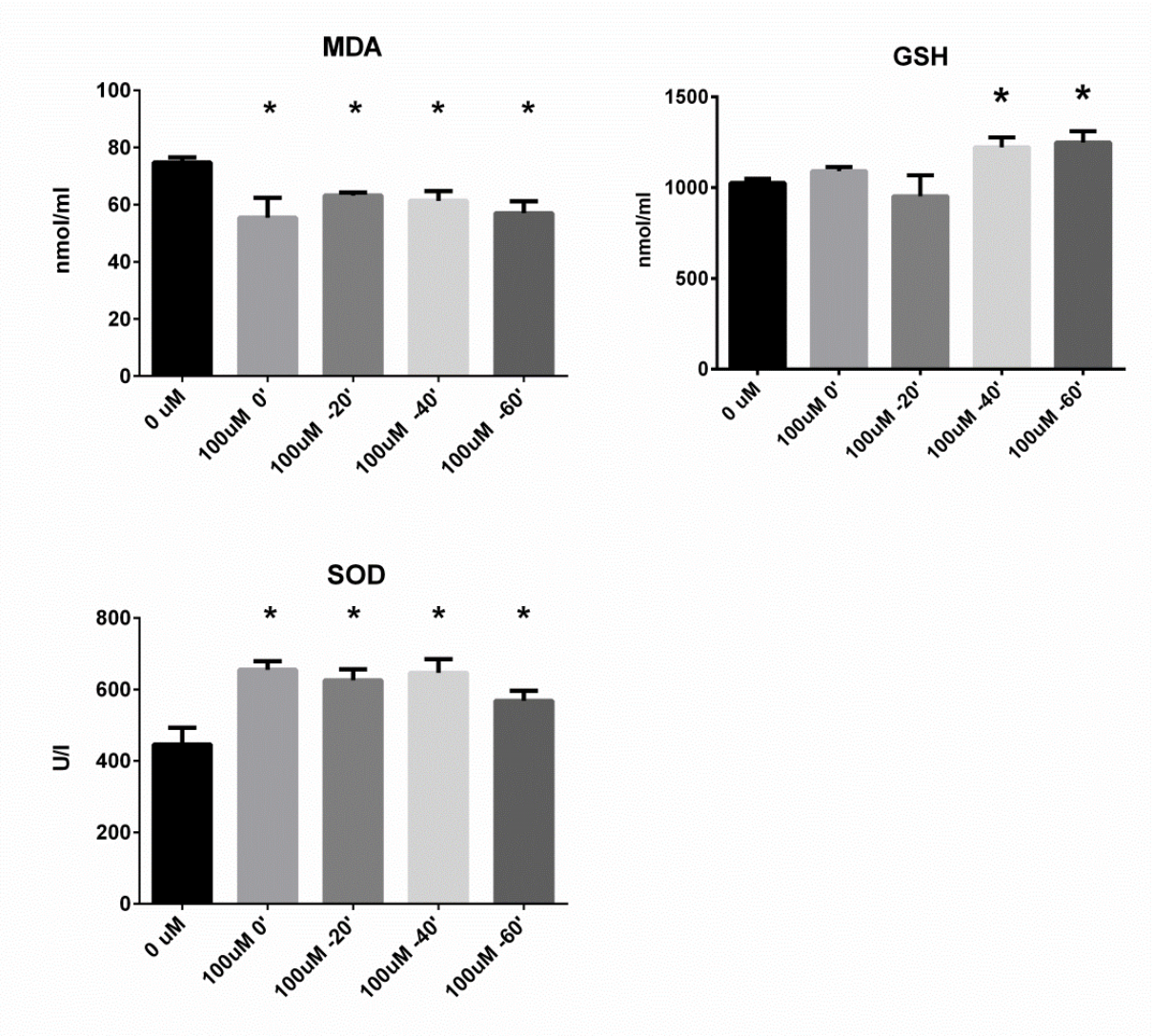


Figure 16. Effect of a PPARGA on oxidative stress markers in time course experiment. PPARGA was added 0, 20, 40, 60 minutes before the start of the hourlong reperfusion. * represents statistical significance ($p < 0.05$) as compared to control according to a one-way ANOVA analysis.

4.3.2. Results of dosage experiments I.

In another experiment, PPARGA was injected into the superior mesenteric vein at different concentrations (0, 10, 50, 100, and 500 μM) 20 minutes before reperfusion. The PPARGA treatment efficiently diminished the level of MDA when injected to deliver a final concentration within the rats of 10, 50, 100, and 500 μM **(Figure 17)**. These results demonstrate that PPARGA is a potent inhibitor of oxidative stress responses during the ischemia-reperfusion process at concentrations as low as 10 μM. In addition, administration of PPARGA at 100 and 500 μM resulted in an increase in SOD activity **(Figure 17)**. Taken together, these results demonstrate that PPARGA is an effective inhibitor of oxidative stress during the ischemia-reperfusion process at levels as low as 10 μM. Consistent with the results from Figure 12, administration of PPARGA at 50, 30 100, and 500 μM 20 minutes before reperfusion resulted in a decrease in the levels of reduced GSH levels. These results demonstrate that PPARGA may be used more effectively as an inhibitor of oxidative stress during the ischemia-reperfusion process when administered more than 20 minutes (e.g., about 30 to 60 minutes such as about 40 minutes) prior to reperfusion.

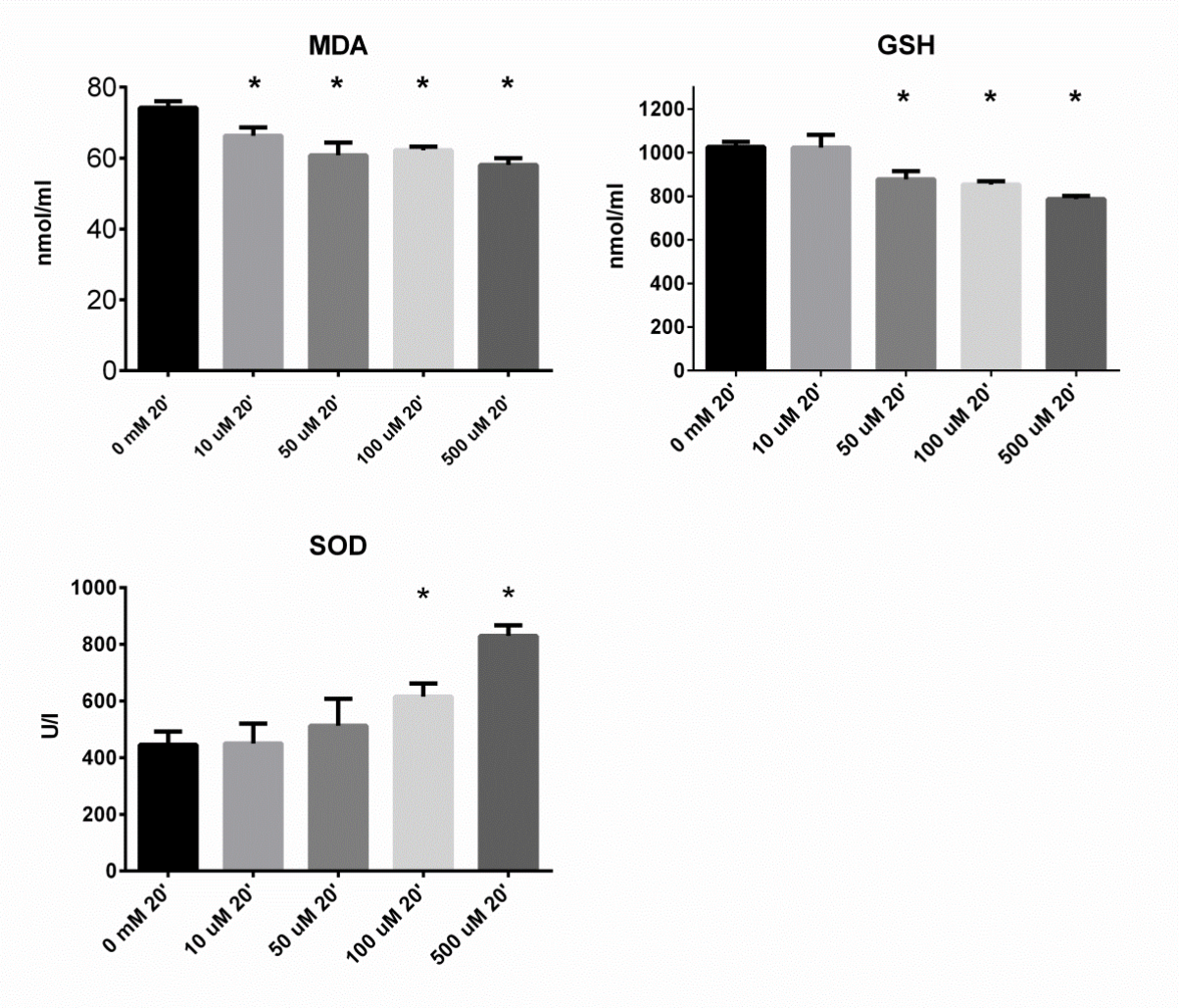


Figure 17. Effect of PPARGA concentration series on oxidative stress markers. PPARGA was added 20 minutes before the start of the hourlong reperfusion. * represents statistical significance ($p < 0.05$) as compared to control according to a one-way ANOVA analysis.

4.3.3. Results of dosage experiments II.

In another experiment, PPARGA was injected into the superior mesenteric vein at different concentrations (0, 10, 50, 100, and 500 μM) 40 minutes before reperfusion. The PPARGA treatment efficiently diminished the level of MDA when injected to deliver a final concentration within the rats of 100 and 500 μM **(Figure 18)**. In addition, administration of PPARGA at 50, 100, and 500 μM resulted in an increase in the levels of reduced GSH levels. Administration of PPARGA at 10, 100, and 500 μM also resulted in an increase in SOD activity (Figure 14). Further, the gene expression pattern of SOD correlated with the SOD activity results as samples for gene expression analysis taken from renal tissue exhibited increased levels of SOD mRNA expression as the amount of PPARGA injected 40 minutes before reperfusion increased **(Figure 19)**. Administration of PPARGA at 10, 50, 100, and 500 μM resulted in an increase in the levels of SH **(Figure 18)**. Thiols can help aerobic cells maintain their reducing state in an oxidizing environment. Thus, higher total thiol levels can indicate a greater reducing state of the cell. Administration of PPARGA 40 minutes before reperfusion also resulted in increased expression of PPARG mRNA **(Figure 20)**. Taken together, these results confirm that PPARGA is an effective inhibitor of oxidative stress during the ischemia-reperfusion process at levels as low as 10 to 50 μM.

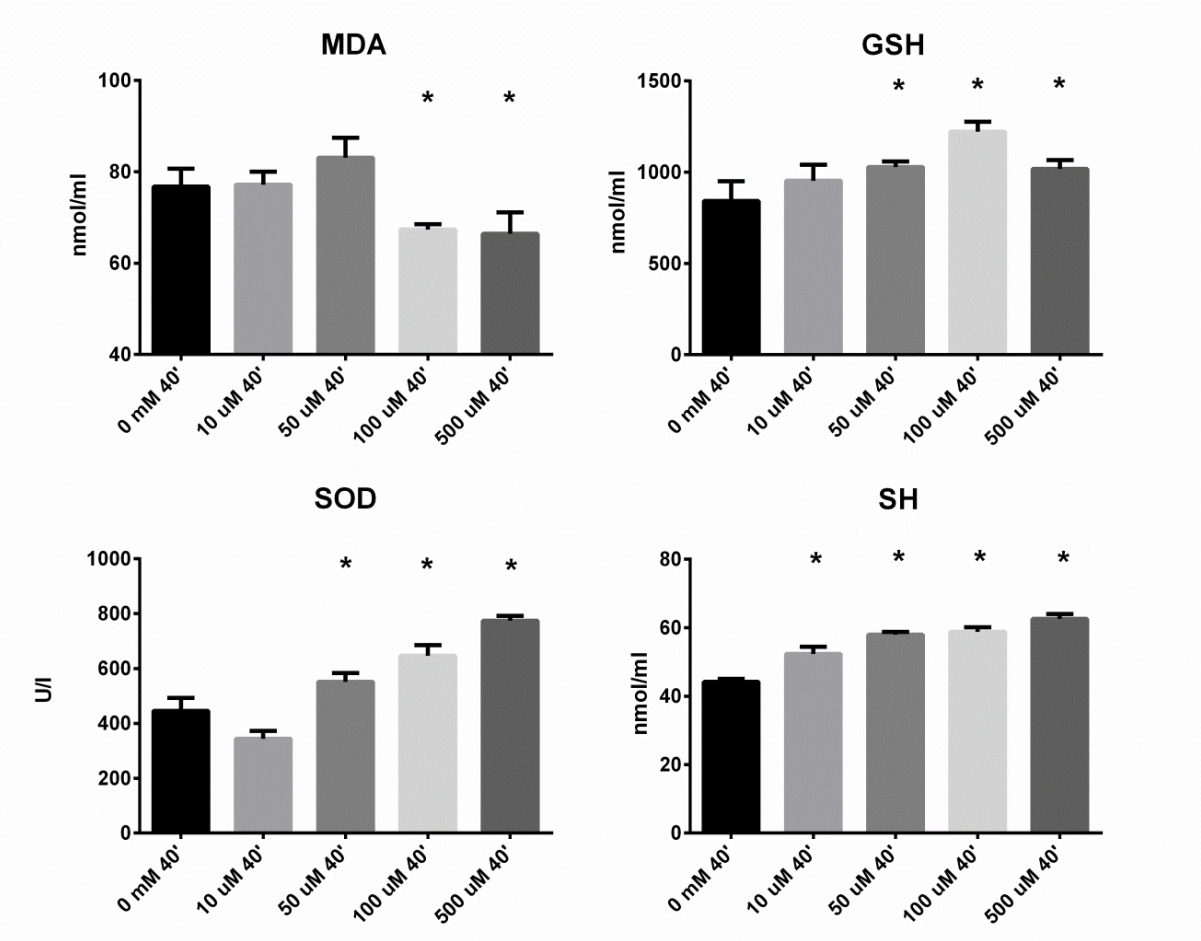


Figure 18. Effects of PPARGA concentration series on oxidative stress markers. PPARGA was added 40 minutes before the start of the hourlong reperfusion. * represents statistical significance ($p < 0.05$) as compared to control according to a one-way ANOVA analysis.

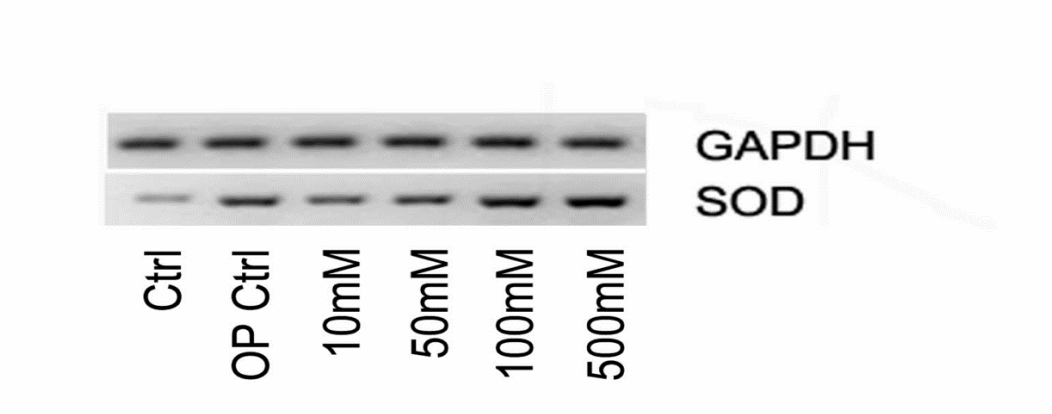


Figure 19. Effect of a PPARGA concentration series on expression of SOD gene. Ctrl: negative control without operation. OP Ctrl: control with operation.

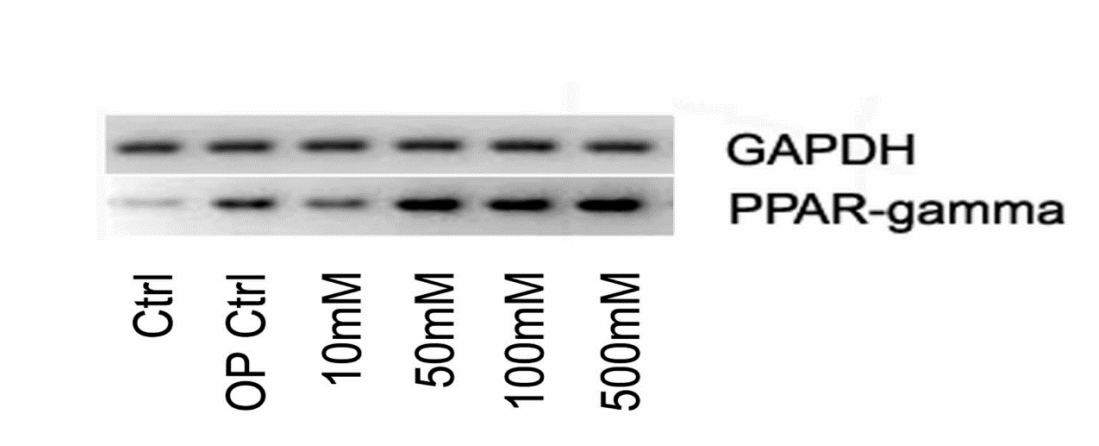
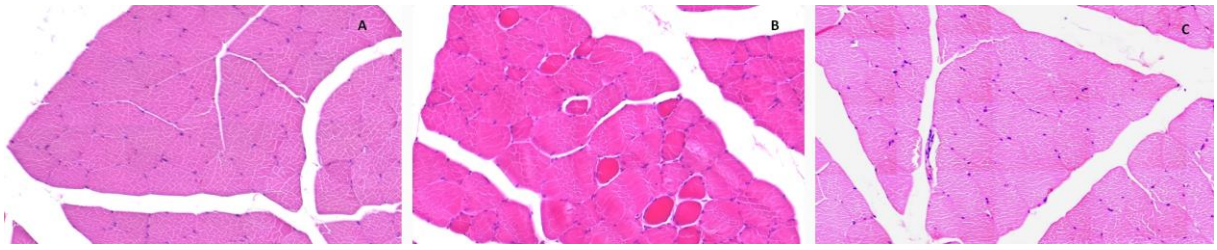


Figure 20. Effect of a PPARGA concentration series on the expression of PPARG gene. Ctrl: negative control without operation. OP Ctrl: control with operation.

4.3.4. Histological results

A histological assessment was performed to confirm that administration of PPARGA reduces ischemia-reperfusion injury in vivo. Revascularization of ischemic skeletal muscle during the reperfusion treatment can result in local and systematic complications like limb edema, skeletal muscle necrosis, pulmonary edema, and compartment syndrome. Skeletal muscle was used herein as a model to study the impacts of ischemia reperfusion injury in a histological assessment since its high metabolic activity makes it extremely susceptible to reperfusion injury even after a brief period of ischemia. In the sham operated control rats (without ischemia-reperfusion treatment), the physiological fiber architecture was intact **(Figure 21A)**. Muscular fibers were polygonal shape, contained peripherally placed nuclei, and exhibited a tightly packed structure **(Figure 21A)**. In ischemic reperfused operated controls, however, many of the numerous fibers lost their internal structure (e.g., round shape, small diameter). About 20 percent of the examined fibers were affected. In addition, there were signs of cellular vacuolizations, and extracellular edema formation **(Figure 21B)**. PPARGA treatment noticeable helped to preserve of the normal tissue and cell architecture with minimal extracellular edema (e.g., minimal fiber separation) and a near complete absence of intercellular vacuolization as compared to the ischemic reperfused operated controls **(Figure 21C)**. These results demonstrate that PPARGA can be administered to a mammal to reduce the severity of ischemia-reperfusion injury.



Figures 21. A-C are photographs of skeletal muscle morphology shown in crosssections stained with hematoxylin and eosin: (A) a sham operated control without ischemia-reperfusion treatment, (B) ischemic reperfused operated control, and (C) PPARGA-treated ischemic-reperfused tissue, where PPARGA was injected intravenously at a final concentration of 100 µM in the 20th minute of a one-hour long ischemic period, which was followed by a one-hour reperfusion.

4.4. Discussion

In this study we aimed to investigate the effects of a PPARGA on ischemia reperfusion injury in bilateral hind limb ischemia rat model. We performed three experiments – one time course and two dosage experiments – to evaluate the effective dosage of the PPARGA and the best time point for administration of it. The PPARG is a member of the nuclear receptor superfamily. PPARs are ligand-dependent transcription factors that bind to specific peroxisome proliferators response elements at the enhancer sites of regulated genes [51]. They are implicated in adipocyte differentiation, insulin sensitivity and inflammatory processes [52, 53], and also down-regulate proinflammatory mediators in macrophages, mainly by inhibiting transcription of NF-kB-dependent inflammatory genes [54, 55, 56]. Emerging evidence indicates that PPARG activation can regulate inflammatory responses, included inflammatory disorders of the central nervous system, inhibiting expression of a variety of pro-inflammatory molecules by a mechanism termed receptor-dependent transrepression [59].

We used in our investigations a natural, non-synthetic PPARGA. We found that the administration of PPARGA can reduce severity of the ischemia reperfusion injury through decreasing the systemic inflammatory response. Beneficial effects of PPARG agonists on ischemia reperfusion injury have been previously documented in the intestine [60, 61, 62], lung [63], heart [64, 65, 66], kidney [67] and, more recently, also in the brain [68, 69].

In the time course experiments we have found that MDA decreased, the GSH levels increased when PPARGA was administered 40 or 60 minutes prior to reperfusion and SOD enzyme activity increases at all time points tested. These results also demonstrate that PPARGA is effective when administered at the time of reperfusion or when administered before reperfusion (e.g., 20, 40, or 60 minutes before reperfusion).

In the first dosage experiment we found that PPARGA is a potent inhibitor of oxidative stress responses during the ischemia reperfusion process at concentrations as low as 10 µM. In addition, administration of PPARGA at 100 and 500 µM resulted in an increase in SOD activity. Consistent with the results from time course experiment, administration of PPARGA at 50, 100, and 500 µM 20 minutes before reperfusion resulted in a decrease in the levels of reduced GSH levels. These results demonstrate that PPARGA may be used more effectively

as an inhibitor of oxidative stress during the ischemia-reperfusion process when administered more than 20 minutes prior to reperfusion.

In the second dosage experiment we found that PPARGA treatment efficiently diminished the level of MDA when injected to deliver a final concentration within the rats of 100 and 500 μM. These results confirm that PPARGA is an effective inhibitor of oxidative stress during the ischemia reperfusion process at levels as low as 10 to 50 μM.

Mehta et al. [68] reported that PPARG agonists diminished reactive oxygen species generation induced by angiotensin II and TNF-alpha, in human coronary artery endothelial cells. Pioglitazone pre-treatment has also been shown to inhibit reactive oxygen species production in cardiac fibroblasts exposed to hypoxia/reoxygenation [69] and to reduce renal oxidative stress in obese rats [70]. Mitochondria are the major source of reactive oxygen species, which are mainly generated at complexes I and III of the respiratory chain [71]. There is now evidence indicating that rosiglitazone and pioglitazone exert direct and rapid effects on mitochondrial respiration, inhibiting complex I [72] and complex III [73] activity. As PPARG agonists partially disrupt the mitochondrial respiratory chain, both electron transport and superoxide anion generation are affected. Moreover, a novel mitochondrial target protein for PPARG agonists (“mito - NEET”) has recently been identified [74]. MitoNEET was found associated with components of complex III, suggesting how PPARG agonists binding to mitoNEET could selectively block different mitochondrial targets. PPARG agonists' ability to influence mitochondrial function might contribute to their inhibitory effects on reactive oxygen species generation evoked by ischemia reperfusion.

4.5. Conclusion

Our investigation results showed that the administration of a PPARGA can decrease the ischemia reperfusion injury in bilateral acute hind limb ischemia rat model. The time course and the dosage experiments showed that PPARGA can reduce the MDA, increase the GSH, SH and the enzyme activity of SOD. In the time course experiment we found that PPARGA is effective when administered at the time of reperfusion or when administered before reperfusion. In dosage experiments we found that PPARGA may be used more effectively as

an inhibitor of oxidative stress during the ischemia reperfusion process at levels as low as 10 to 50 μM when administered more than 20 minutes prior to reperfusion.

The clinical importance of this study is that PPARGAs seem to be an effective, quick and simple method to decrease the reperfusion damages after surgery. So in case of patients suffering from arterial occlusive vascular disease, the administration of this drug during revascularization surgery before the complete restoration of blood flow is worthy of considering.

5. EFFECTS OF PHOSPHODIESTERASE INHIBITION ON INFRARENAL ABDOMINAL AORTIC ISCHEMIA REPERFUSION: ROLE OF PENTOXIFYLLINE

5.1. Introduction

Despite significant research efforts and aggressive treatment strategies, in case of acute ischemia the extent of ischemia reperfusion injuries after revascularization surgery remains high. The severity of these injuries depends on the ischemic time, the collateral circulation of the affected limb, the localization of the occlusion and the general state of the affected tissues. In reperfusion injury the developing local than systemic inflammatory response plays a crucial role in severe tissue injury and organ dysfunction and may develop into multiple organ dysfunction syndrome-MODS. In the early reperfusion, when the molecular oxygen appears in the cell, the – xanthine oxidase catalyzed – hypoxanthine-xanthine conversion will produce a mass of superoxide radicals. Rapid generation of ROS by activated endothelial cells, neutrophils (NADPH oxidase, myeloperoxidase-MPO), lipid mediators (platelet activating factor-PAF, leukotriene B4-LTB4) are main pathways in the process of inflammatory response. During reperfusion the superoxide radicals neutralize the nitrogen monoxide-NO produced by endothelial cells. Reduced NO availability leads to augmented expression of cellular adhesion molecules, vasoconstriction, formation of micro-thrombi, induction of local inflammation, leukocyte infiltration. The nuclear factor kappa B (NFkB) is a transcription factor which determines an up-regulation of the genes responsible of the production of molecules of cellular adhesion. [75] These molecules favour the adhesion of leukocytes to the endothelium and possibly the migration within the cells. [76] These mechanisms can lead to the so-called “no-reflow phenomenon”. [77]

Pentoxifyllin (1-[5-oxohexyl]-3,7-dimethylxanthine),(PTX) a xanthine-derived non-specific phosphodiesterase (PDE) inhibitor, has been used for the treatment of intermittent claudication in patients suffering from peripheral and cerebrovascular disease. [78] Through its hemorheologic properties, PTX can modify the conformation of red blood cells and improve the microcirculatory blood flow in chronic arterial insufficiency. On the other hand

PTX has been used in the attenuation of the inflammatory response too. Recent studies have focused on the anti-inflammatory effects of PTX, more specifically, the neutrophils.
We hypothesized that single-shot, increased dose of PTX treatment in conjunction with its known hemorheological effects decreases the developing ischemia-reperfusion injury and can attenuate the local and systemic inflammatory response.

5.2. Materials and methods

5.2.1. Animal model

50 male albino Wistar rats, weighed between 200-250 g were used in the present study from Charles River Breeding Laboratories (Hungary, Isaszeg). The animals were housed in individual cages in a temperature (25 ± 2˚C), light controlled (12 hours light-dark cycle) and air-filtered room with free access to food and water. Food was withdrawn 12 hours prior to experiment. The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

5.2.2. Aortic ischemia reperfusion model

The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (500 mg / 10 ml) and diazepam (10 mg / 2 ml). The ratio was 1:1 (0.2 ml / 100 g = 5 mg ketamine + 0.5 mg diazepam / 100 g) and the animals were placed on a heated pad. The skin was disinfected and a midline laparotomy was performed. 2 ml of warm saline was injected into the abdominal cavity to help maintain the fluid balance. The inferior mesenteric vein was catheterized for collecting blood samples, fluid equilibration and supplemental anesthetic. The abdominal aorta was exposed by gently deflecting the intestine loops to the left. After fine isolation of the infrarenal segment, an atraumatic microvascular clamp was placed on the aorta for 60 minutes. The abdomen was then closed and the wound was covered with warm, wet compress to minimize heat and fluid losses. The microvascular clamp was then removed and the infrarenal abdominal aorta was reperfused for 120

minutes. Aortic occlusion and reperfusion was confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta.

5.2.3. Administration of pentoxifyllin

Animals in the treated groups received intravenous bolus of PTX (50mg/kg) half an hour before the reperfusion. Control animals received only normal saline solution. The dosage based on data from literature.

5.2.4. Protocol of ischemic postconditioning

Those groups wherein the animals underwent ischemic postconditioning, after the ischemic phase intermittent 15 seconds reperfusion – 15 seconds ischemic periods were applied four times.

5.2.5. Experimental groups

Rats were divided into five groups (10 rats in each group). In the control group a midline laparotomy was performed for three hours. Normal saline solution was administered to the animals intravenously 30 minutes before the reperfusion phase (control). The infrarenal abdominal aorta in the second group was closed for 60 minutes and then 120 minutes of reperfusion followed (IR). Rats in the third group underwent a 60 minutes of ischemia, after the ischemic phase postconditioning was performed followed by a 120 minutes reperfusion phase (IR+PC). In the fourth group 60 minutes of ischemia was performed, 30 minutes before the reperfusion PTX was administered to the animals and then 120 minutes of reperfusion is followed (IR+PTX). Rats in the fifth group underwent a 60 minutes of ischemia, 30 minutes before the reperfusion PTX was administered to the animals, after the ischemic phase postconditioning was performed followed by 120 minutes of reperfusion (IR+PC+PTX). **(Fig.22.)**

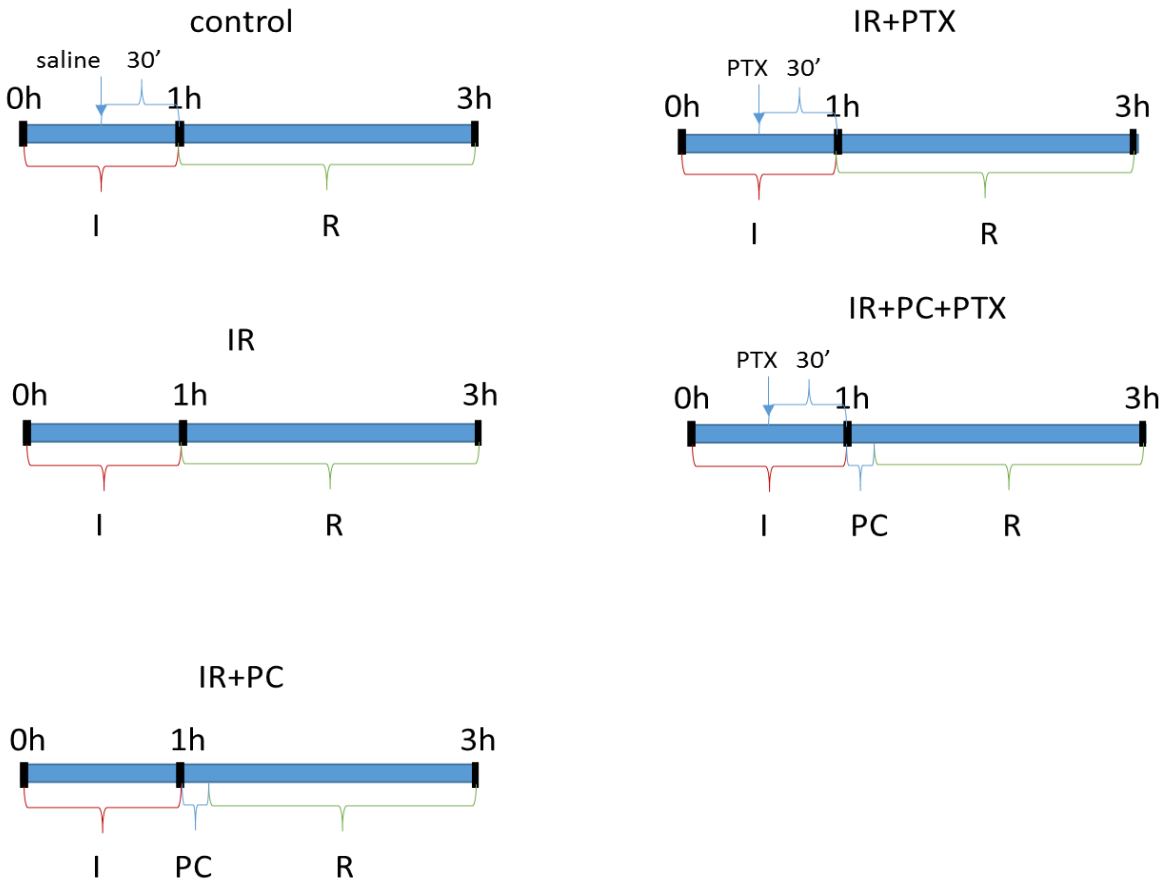


Fig.22. Investigation groups. I: ischemia, R: reperfusion, PC: postconditioning, PTX: pentoxifylline.

Peripheral blood samples and biopsy from quadriceps muscle were collected from the animals at the end of the reperfusion phase. The serum and tissue samples were harvested and stored at minus 78˚C until biochemical assays.

5.2.6. Analysis of oxidative stress parameters

Measurement of MDA: Malondialdehyde is a marker for the quantification of lipid peroxidation in cell membranes. MDA was determined in anticoagulated whole blood, by photometric method of Placer, Cushman and Johnson. [32]

Measurement of reduced glutathione and plasma thiol-groups: Reduced glutathione is the predominant low-molecular-weight thiol in cells. Because of the cysteine residue GSH is readily oxidized nonenzymatically to glutathione disulfide by electrophilic substances. GSH concentrations reduce markedly in response to protein malnutrition and oxidative stress. [33] GSH and plasma SH levels were determined in anticoagulated whole blood EDTA by Ellman's reagent according to the method of Sedlak and Lindsay. [34]

For measuring of SOD activity in serum we used Superoxide Dismutase Assay Kit (Trevigen Inc., Gaithersburg, USA), following the manufacturers protocol. This method determines the free i.e. biological active SOD activity.

5.2.7. Serum TNF-alpha and IL-6 quantification

For measuring TNF-alpha and IL-6 concentration in serum we used Rat TNF-alpha and Rat IL-6 ELISA kit (R&D Systems, Inc., Minneapolis, USA), following the manufacturers protocol. These methods determine the free i.e. biological active TNF-alpha and IL-6 concentrations.

5.2.8. Statistical analysis

All values are expressed as means ± SEM. Differences between the variances of the groups were assessed with one-way analysis of variance (ANOVA) and when the results were significant we used adequate post-hoc tests for multiple comparisons. For comparing the treated groups to the control group we performed in case of each investigated parameters Dunnett's test. We used Sidak post-hoc test for comparisons across multiple different

groups. Multiple comparisons tests resulted in adjusted p-values, each p-value is adjusted to account for multiple comparisons. We performed five-five comparisons (Dunnett's and Sidak) per investigated parameter. T-tests were performed independently to show the differences between the investigated groups. Data were considered significant when p-value was less than 0.05.

5.3. Results

5.3.1. Plasma malondialdehyde levels

We measured in an in vivo animal model the values of malondialdehyde plasma-level indicating membrane damage and lipid peroxidation. MDA concentration was significantly higher in all groups (IR, IR+PC, IR+PTX, IR+PC+PTX) comparing to the control group (79.39 ± 0.64; 68.16 ± 0.62; 70.97 ± 1.23; 65.52 ± 0.98 nmol/ml vs. 61.12 ± 1.75 nmol/ml / p <0.0001; p =0.002; p <0.0001; p =0.0285). Our data showed significantly lower MDA concentrations in IR+PC, IR+PTX and IR+PC+PTX groups comparing to the IR group (68.16 ± 0.62; 70.97 ± 1.23; 65.52 ± 0.98 nmol/ml vs. 79.39 ± 0.64 nmol/ml / p <0.0001; p <0.0001; p <0.0001). In the IR+PC+PTX group we found significantly lower MDA concentrations than in IR+PTX group (65.52 ± 0.98 nmol/ml vs. 70.97 ± 1.23 nmol/ml / p =0.0065). **(Fig. 23.)**

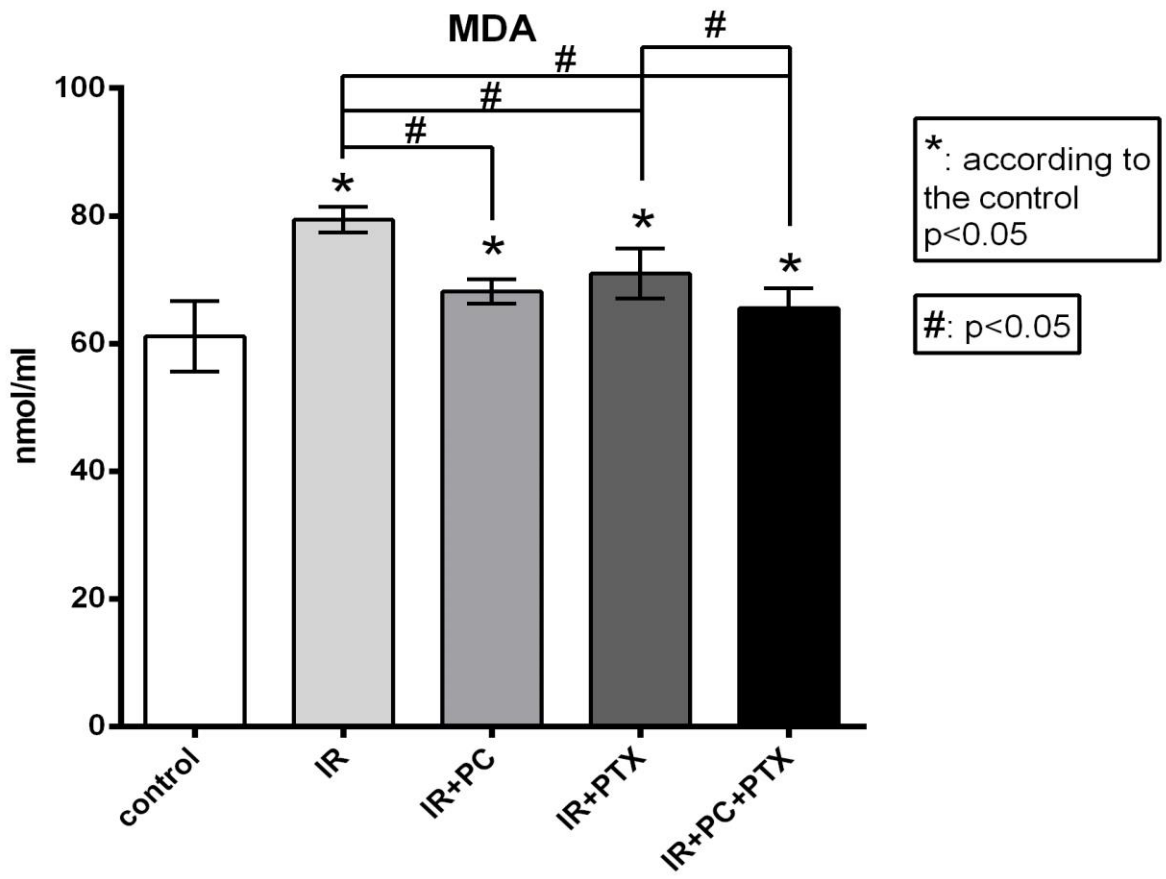


Fig.23. Malondialdehyde concentrations in the experimental groups. MDA signs the severity of lipidperoxidation. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

5.3.2. Reduced glutathione levels (GSH)

The values of reduced glutathione levels were significantly lower in two groups (IR, IR+PTX) comparing to the control group (725.1 ± 11.26; 808.6 ± 14.72 nmol/ml vs. 877.1 ± 20.7 nmol/ml / p <0.0001; p =0.033). Our data showed significantly higher concentrations in IR+PC, IR+PTX and IR+PC+PTX groups comparing to IR group (822.8 ± 23.13; 808.6 ± 14.72; 830.6 ± 17.3 nmol/ml vs. 725.1 ± 11.26 nmol/ml / p =0.0018; p =0.0097; p =0.0007). **(Fig. 24.)**

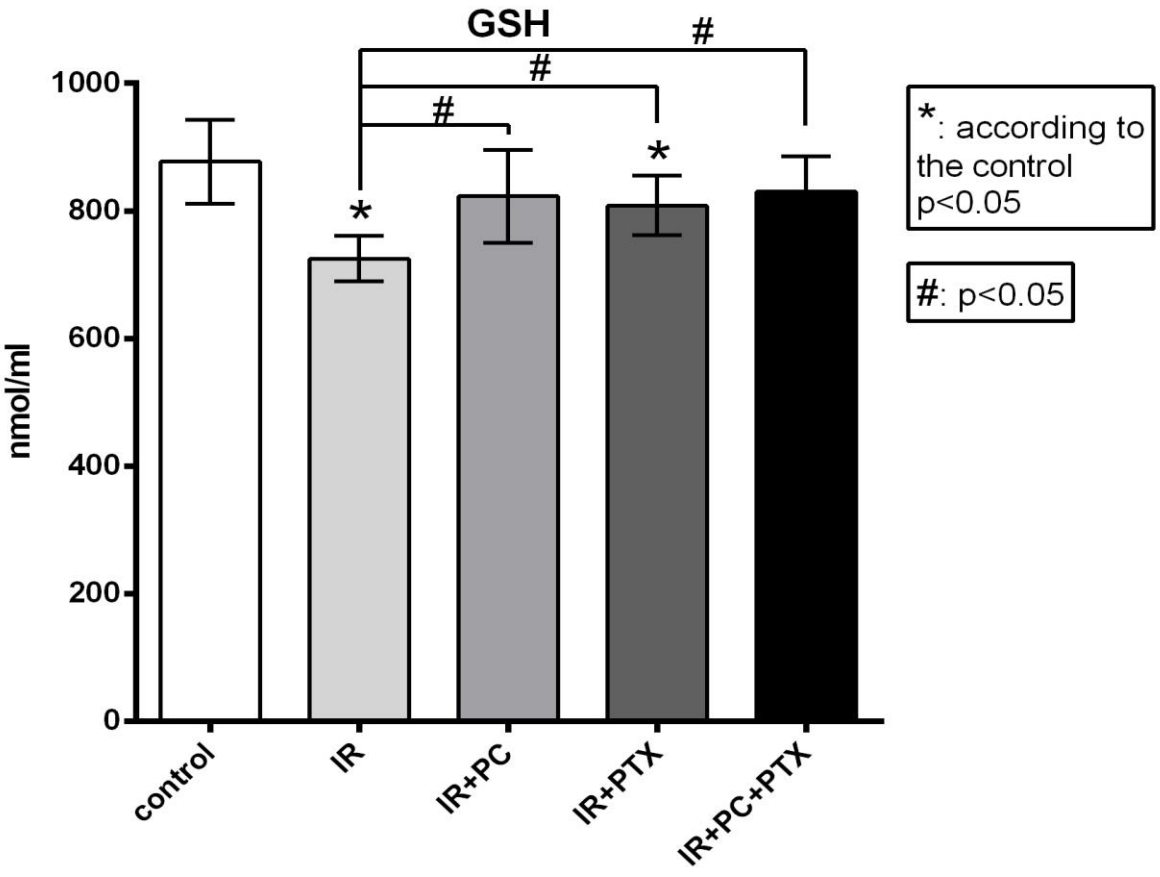


Fig.24. Plasma concentrations of reduced glutathione in the investigated groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

5.3.3. Plasma thiol groups (-SH)

We detected in the IR group significantly lower level of –SH comparing to control group (42.09 ± 2.15 nmol/ml vs. 54.02 ± 2.68 nmol/ml / p =0.003). There was no significant difference in –SH level between other groups. **(Fig. 25.)**

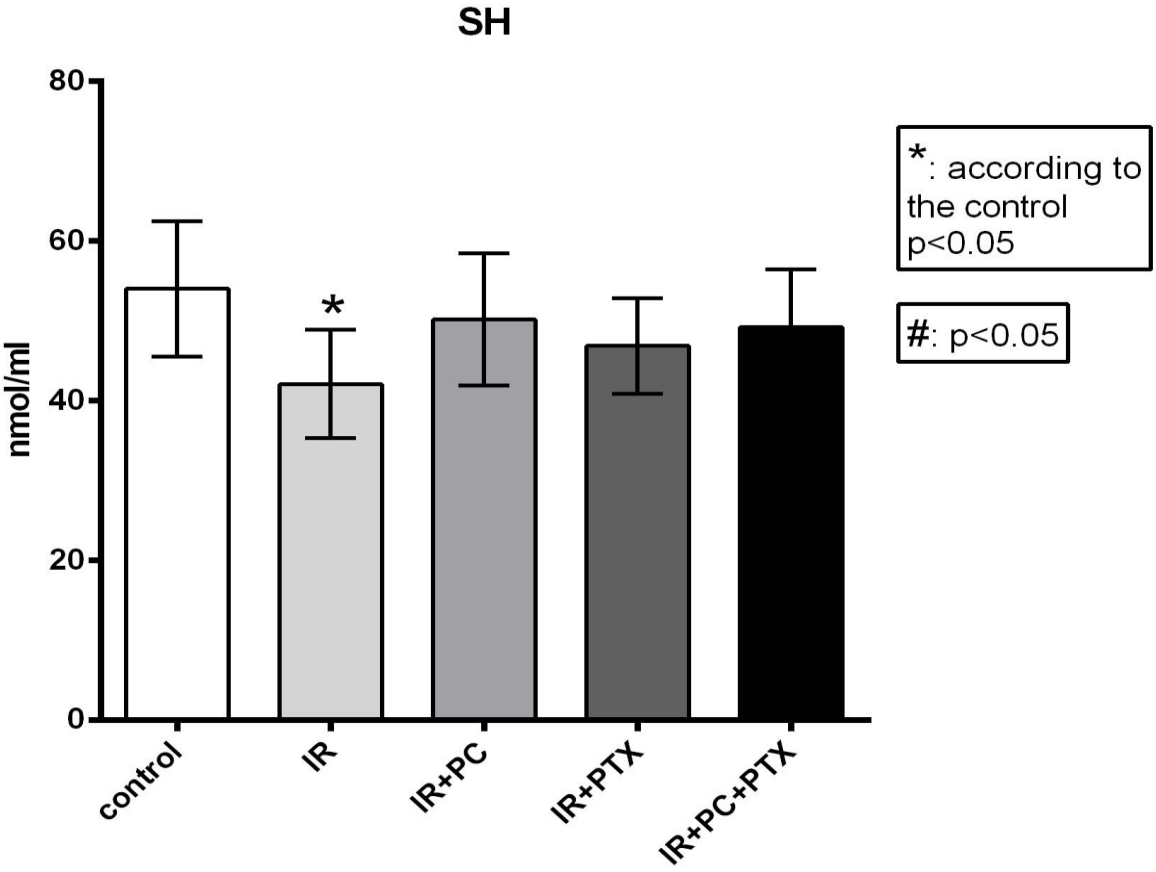


Fig.25. Concentrations of –SH groups in the plasma. *: p<0.05 vs. control; error bars: SD

3.3.4. Enzyme activity of superoxide dismutase (SOD)

We have detected in two investigated groups significantly elevated (IR+PC, IR+PC+PTX) and in one group significantly lower (IR) SOD activity comparing to the control group (1088 ± 42.1; 1113 ± 52.8 U/l vs. 893.7 ± 32.6 U/l / p =0.0026; p =0.0006; 533.8 ± 17.4 U/l vs. 893.7 ± 32.6 U/l / p <0.0001). In IR+PC, IR+PTX and IR+PC+PTX groups we have detected significantly elevated SOD activity comparing to IR group (1088 ± 42.1; 952.4 ± 34.1; 1113 ± 52.8 U/l vs. 533.8 ± 17.4 U/l / p <0.0001 in all three comparisons). In IR+PC+PTX group we found significantly elevated SOD activity than in IR+PTX group (1113 ± 52.8 U/l vs. 952.4 ± 34.1 U/l / p =0.02). **(Fig. 26.)**

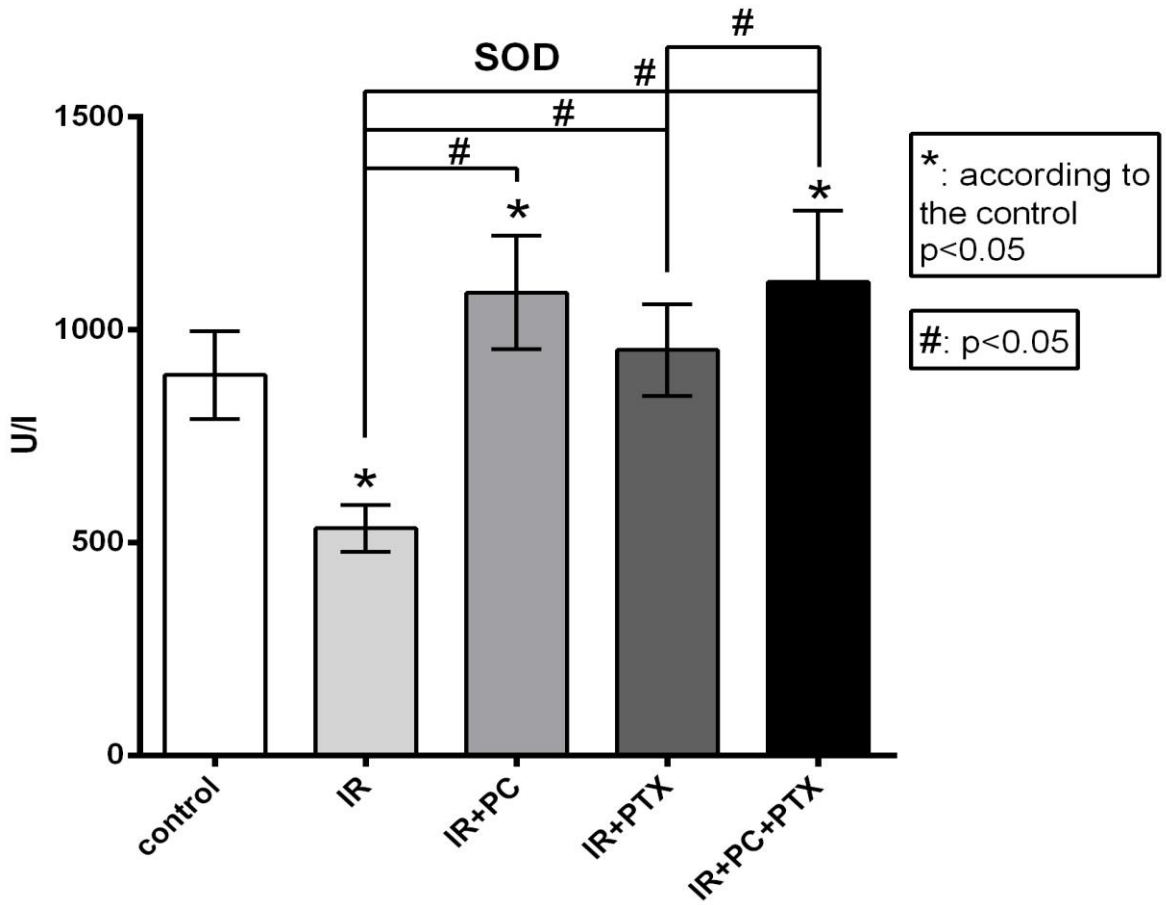


Fig.26. Enzyme-activity of superoxide dismutase in the investigated groups.; *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

5.3.5. Serum TNF-α levels

In the study we measured the TNF-α levels in the groups. The values were significantly higher in IR group than in the control group (21.9 ± 0.49 pg/ml vs. 18.4 ± 0.3 pg/ml / p <0.0001). In IR+PC, IR+PTX and IR+PC+PTX groups we detected significantly lower values comparing to the IR group (19.7 ± 0.3; 18.6 ± 0.4; 19.05 ± 0.3 pg/ml vs. 21.9 ± 0.5 pg/ml / p =0.0002; p <0.0001; p <0.0001). **(Fig. 27.)**

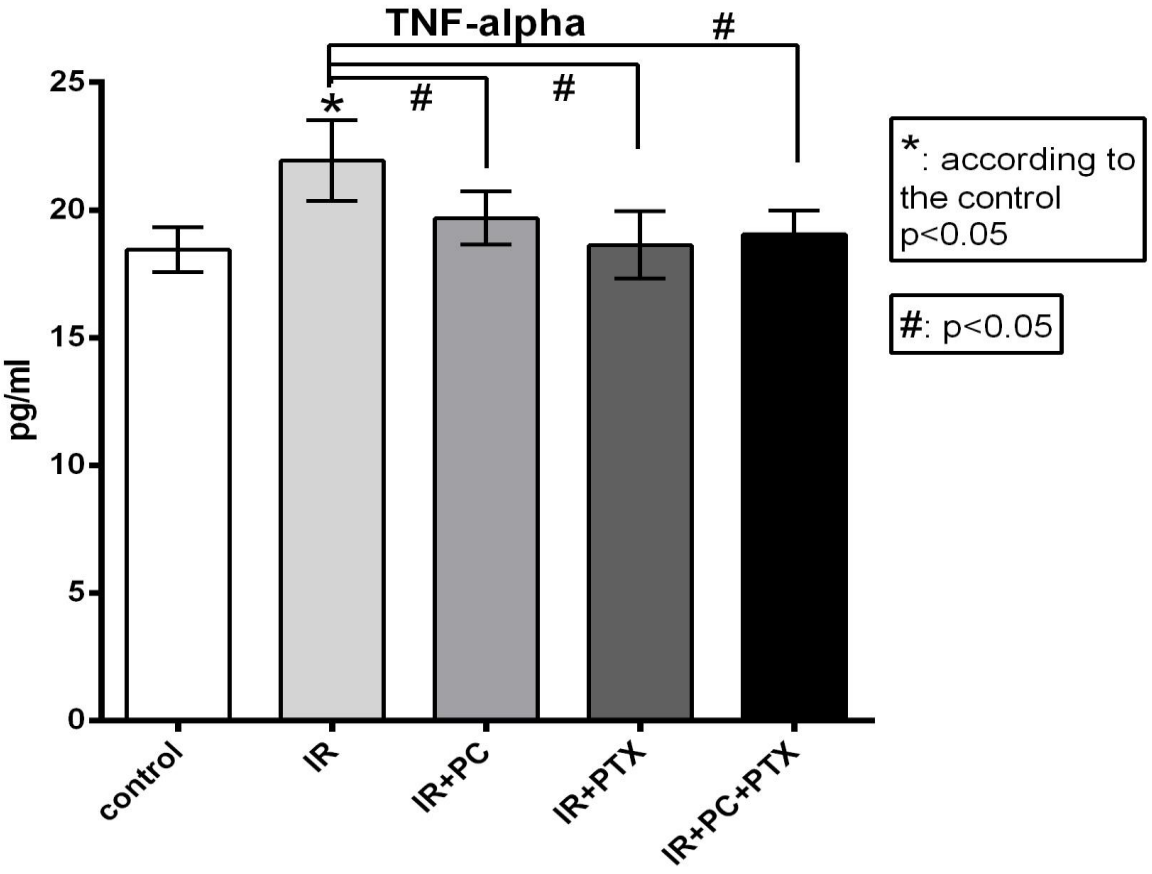


Fig.27. TNF-alpha concentrations shows the grade of inflammatory response in the groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

3.3.6. Serum interleukin-6 (IL-6)

We investigated the serum IL-6 levels in our groups. The values were significantly higher in IR group, than in the control group (144.3 ± 4.2 pg/ml vs. 109.3 ± 1.9 pg/ml / p =0.002). We have found significantly lower concentrations in IR+PTX, IR+PC and IR+PC+PTX groups than in IR group (112.9 ± 2.1; 119.9 ± 3; 115.9 ± 2.7 pg/ml vs. 144.3 ± 4.2 pg/ml / p <0.0001; p <0.0001; p <0.0001). **(Fig. 28.)**

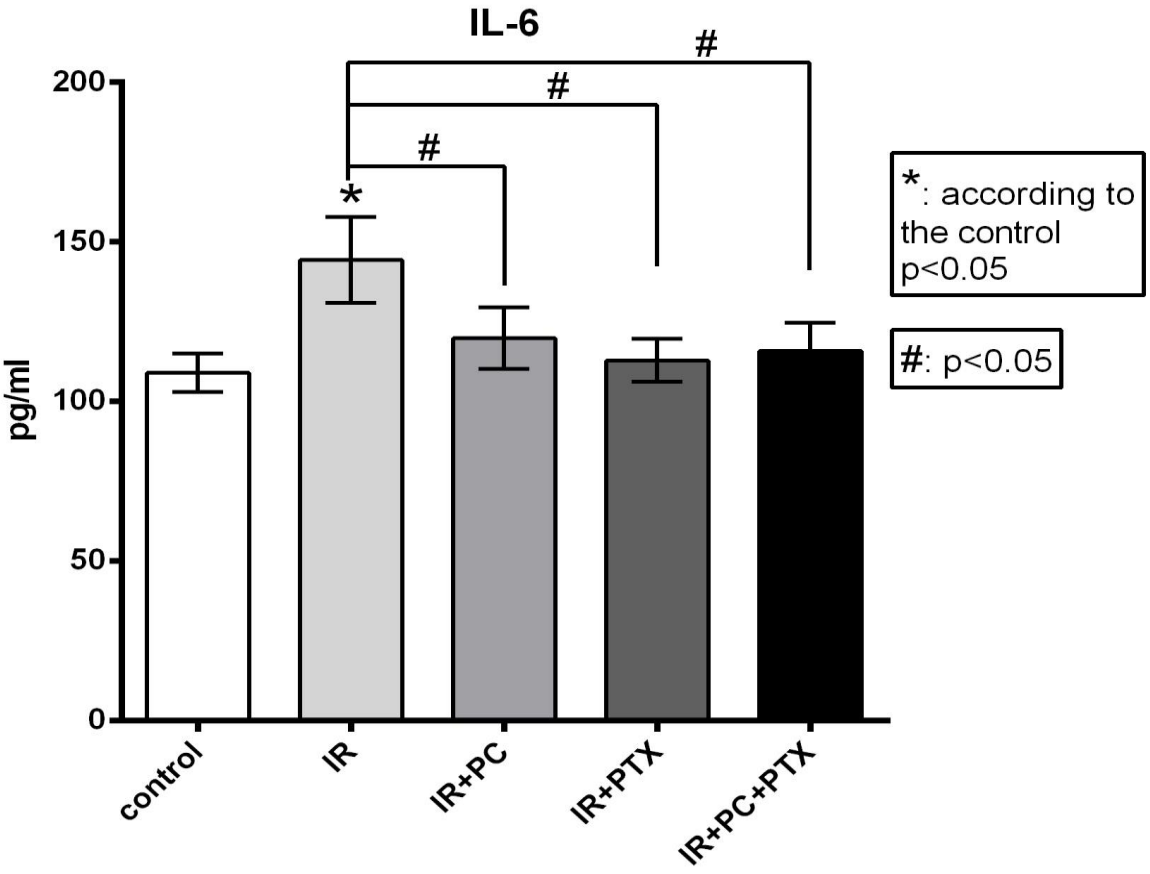


Fig.28. IL-6 plasma-concentrations shows the grade of inflammatoric response in the groups. *: p<0.05 vs. control; #: p<0.05 between the signed groups; error bars: SD

5.4. Discussion

After revascularization procedures we always have to face with severe or less reperfusion injury. Numerous factors can modulate the extent of reperfusion injury including inflammatory response.

PTX a xanthine-derived non-specific PDE inhibitor, has been used for the treatment of intermittent claudication in patients suffering from peripheral and cerebrovascular disease [78]. Through its hemorheologic properties, PTX can modify the conformation of red blood cells and improve the microcirculatory blood flow in chronic arterial insufficiency. On the other hand recently PTX has been used in the attenuation of the inflammatory response too. PTX can decrease the inflammatory process after cardiopulmonary bypass in open-heart surgery, sepsis, and acute respiratory distress syndrome (ARDS) in neonates. PTX exerts multiple beneficial effects on the inflammatory cascade by increasing intracellular cyclic adenosine monophosphate (cAMP) and decreasing TNF-alpha and IL-6 synthesis. [79, 80] An increase in cAMP levels in muscle fibers results in the activation of protein kinase-A-PKA and facilitates synaptic transmission in the mammalian neuromuscular junction (NMJ). Blocking the production of TNF-alpha by PTX takes place by activation of adenyl cyclase and increased levels of intracellular cAMP. This in turn decreases the amount of arachidonic acid that undergoes peroxidation. The overall effect is a decrease in systemic and local concentrations of inflammatory agents such as cyclooxygenase. [81, 82]

NFkB is a transcription factor which plays a double edged sword role in tissue processes. Activation of NFkB is essential for late preconditioning, in which NFkB is involved in the up-regulation if inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) genes. NFkB is also important in reperfusion injury. It contributes to exacerbation of the tissues' lesions sustaining inflammatory reactions. The activation of NFkB is induced by inter alia hydrogen peroxide.

The relationship between transcription factors and PTX has yet to be determined. PTX dose-dependently reduced NFkB subunit nuclear translocation when given lipopolysaccharide (LPS). [83] PTX also diminishes NFkB translocation in activated T lymphocytes. [84] These results suggest that PTX is involved in a common signaling pathway, however, further experimentation is necessary.

In our study we hypothesized that single shot, increased dose of PTX treatment in conjunction with its known hemorheological effects decreases the extent of developing ischemia-reperfusion injury and can attenuate the local and systemic inflammatory response. A recent study has demonstrated that PTX attenuates ischemia reperfusion injury in skeletal muscle and other tissues by decreasing neutrophil adhesion to endothelial cells, ROS production, and platelet activation (PAF). [85] During investigation of oxidative parameters we have found that postconditioning and PTX administration decreased significantly the plasma levels of MDA comparing to the IR group which further decreased in the "co-treated" group. GSH is an endogenous antioxidant. Postconditioning and administration of PTX could significantly moderate the decrease of GSH level in the groups. Enzyme activity of SOD was significantly higher both postconditioned and PTX-administered groups comparing to IR group. Beside the hemorheological effects, the additive beneficial pathway of PTX can be the anti-inflammatory effect. Recently El-Ghoneimi et al [86] reported significantly lower levels of serum TNF-alpha and a lower necrotic area in liver tissue in the PTX group. PTX has been shown to downregulate the synthesis of proinflammatory mediators like IL-6, improve microvascular hepatic and intestinal blood flow after hemorrhagic shock. [87, 88, 89, 90] During investigation of inflammatory response we performed TNF-alpha and IL-6 ELISA. We found that administration of PTX could decrease significantly both the TNF-alpha and the IL-6 concentrations in plasma. The degree of these decreases could beyond the decrease observed in the postconditioned groups. Our data seems to be confirmed the recent findings, that PTX has anti-inflammatory effects through inhibition of TNF-alpha and IL-6 formation and attenuation of neutrophil adhesion to endothelial cells and platelet activation. As TNF-alpha is an inducer of the inflammatory cascade, it also acts as trigger to the extrinsic pathway of apoptosis. [91,92] So decreased TNF-alpha concentration can lead to attenuation of apoptosis as well.

5.5. Conclusion

Our results showed that administration of PTX can decrease the extent of ischemia reperfusion injuries including the inflammatory response through its hemorheological- and recently described anti-inflammatory effects. In our study the administration of PTX could

reach almost the same protection like ischemic postconditioning. The results of the investigated inflammatory mediators could support the finding, that PTX has anti-inflammatory or immunmodulating effects as well. So the clinical importance of this investigation is the possible beneficial effects of PTX on ischemia-reperfusion injury due to its hemorheological and anti-inflammatory effects.

6. NOVEL FINDINGS

In the first series of our investigations we observed the effects of inhibition of GST by ethacrynic acid and ischemic postconditioning after aortic occlusion-induced reperfusion injury in an experimental animal (rat) model.
In the second phase of our investigation we examined the effects of a non-synthetic PPARGA on ischemia reperfusion damages in bilateral hind limb acute ischemia rat model.
In the third series of our study we examined the effects of phosphodiesterase inhibitor pentoxifylline on reperfusion induced inflammatory response in case of infrarenal aortic ischemia and reperfusion.

We have 6 important observations in the study:

1. Firstly we demonstrated in in-vivo animal experiment that pharmacological inhibition of GST by EA could augment the ischemia reperfusion injury after infrarenal aortic occlusion. Furthermore GST inhibition was associated with different activation of MAP kinases regulating pro- and antiapoptotic pathways under stress conditions.

2. We described first in in-vivo experiment that the protective effect of ischemic postconditioning could not develop in case of GST inhibition by EA. Hypoxia and reoxygenization was able to decrease the activation of JNK and p38 MAPKs, suggest that modulation of MAPK signaling pathways are involved in the postconditioning-induced protection.

3. Beneficial effects of PPARG agonists on ischemia reperfusion injury have been previously documented in the intestine [60, 61, 62], lung [63], heart [64, 65, 66], kidney [67] and, more recently, also in the brain [66, 69]. We described first the positive effects of a PPARGA on ischemia reperfusion injury in the skeletal muscle in in-vivo animal model.

4. We performed at first time time-course and dosage experiments in in-vivo animal model to explain the most effective timing and dosage of administration of PPARGA to decrease the ischemia reperfusion injury after infrarenal aortic occlusion.

5. We proved in in vivo animal model that PTX has an additional anti-inflammatory effect in case of infrarenal aortic ischemia and reperfusion due to attenuation of TNF-alpha and IL-6 concentration.

6. Single shot, high dose administration of PTX (50 mg/kg) could augment the positive effects of ischemic postconditioning.

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9. LIST OF PUBLICATIONS

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