The role of glutathione S-transferase in myocardial ischaemic-reperfusion pathways and endogenous adaptation responses

PhD Thesis

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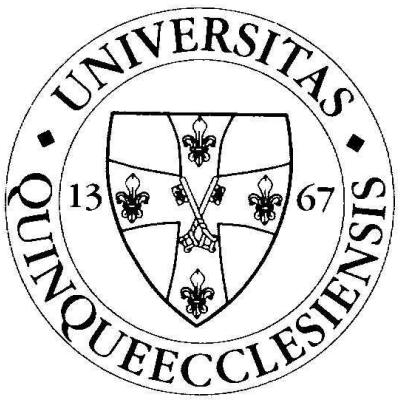

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ABBREVIATIONS

ACCT	Aorta cross clamp time
Akt/PKB	Protein kinase B
AMI	Acute myocardial infarction
AMP	Adenosine monophosphate
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CI	Confidence intervals
CKMB	Myocardial based creatinin kinase
CNV	Copy Number Assay
Cyt C	Cytochrome C
CPB	Cardiopulmonary bypass
DNA	Deoxyribonucleic acid
EA	Ethacrynic acid
ECG	Electrocardiogram
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase-p44/42
GR	Glutathione reductase
GSH	Glutahione
GSK3	Glycogen synthase kinase-3
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GSTM1/GSTM	Glutathione S-transferase Mu
GSTP1/GSTP	Glutathione S-transferase PI
GSTT1/GSTT	Glutathione S-transferase Theta
IL-1	Interleukin 1
IP3	Inositol trisphosphate
IPoC	Ischaemic postconditioning

LAD	Left anterior descending
MPO	Plasma myeloperoxidase
OR	Odds ratio
PC	Ischaemic postconditioning
PI3K	Phosphoinositide-3-kinases
PKB	Protein kinase B
PMI	Perioperative myocardial infarction
I/R	Ischaemia/reperfusion
JNK	c-Jun N-terminal kinase
LCOS	Low cardiac output syndrome
MAPK	Mitogen aktivated protein kinase
MDA	Malondialdehyde
MDS	Myelodysplastic syndromes
MEK	MAPK/ERK kinase; mitogen-activated protein kinase/extracellular signal-regulated kinase
MEKK1	Mitogen-activated protein kinase kinase 1
mPTP	Mitochondrial permeability transition pore
p38	p38 mitogen activating protein kinase
PCR	Polymerase chain reaction
RBC	Red blood cell
RISK	Reperfusion Injury Salvage Kinase
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen radicals
RTK	Receptor tyrosine kinase
SCE	Sister chromatid exchanges
SNP	Single Nucleotide Polymorphism
SOD	Superoxide dismutase
TI	Troponin I
TNF alpha/α	Tumor necrosis factor alpha

1. INTRODUCTION

1.1 MYOCARDIAL ISCHAEMIC/REPERFUSION INJURY

It has been well investigated that oxidative stress following ischaemic-reperfusion injury is a major apoptotic stimulus in many cardiac diseases. Ischemia can be characterized as an inadequate blood flow to a part of the body, caused by constriction or blockage of vessels, formation of thrombus, coronary atherosclerosis or coronary spasm. It is well known that myocardial ischemia results in the loss of contractile function and procedures myocardial damage as a consequence of cell death from both necrosis or apoptosis. The first step during this ischemia is the accumulation of intracellular sodium, hydrogen and calcium ions conclude by tissue acidosis. This decreased pH trigger results an elevated Na+-H+ exchange and Na+-HCO3- transporter, which lead to intracellular sodium and calcium ion overload. This harmful process initiate the activating a crowd of intracellular enzymes including proteases and endonucleases which are important in proapoptotic signaling. The next process is an increased neutrophil adhesiveness and a constant generation of reactive oxygen radicals (ROS) via stimulation of neutrophil oxydative burst. The absence of oxygen causes energy depletion (ATP) with consequent mitochondrial dysfunction and initiates the translocation of Bax, a proapoptotic Bcl2 family member protein, from the cytosol to the outher mitochondrial membrane. The mitochondrium is swelling and induces the efflux of citochrome c via opening of the permeability transition pore into the cytosol where cytochrome c activates effector caspases and initiates apoptosis. Dysfunction of the mitochondrial electron transport system promotes the generation of ROS and development of oxidative stress in the ischemic heart. ROS able to react directly with DNA, cellular lipids, proteins leading to cell death and activation of tumor necrosis factor alpha (TNF-alpha). Catalase, superoxide dismutase (SOD), glutathione peroxidase and repaire enzymes are in the first line of antioxidant protection, but recently among other antioxidant enzymes researches are focus on glutathione S-transferase (GST).

Repair of sufficient oxygen and nutrients can limit the size of the final extent of injury but paradoxically the restoration of normal blood flow to an area of ischemia results in a complex cascade of inflammation and oxidative stress leading to cell necrosis and apoptosis. During this process the vascular endothelium upregulates the production of adhesion proteins and releases leukocyte attractans. These are mostly neutrophils and monocytes which triggers

multiple mediator cascades leading to cytokine and chemokine release, the generation of ROS and the release of proteolytic enzymes from white blood cells and increased vascular permeability. During ischemia, the hydrolysis of ATP via AMP leads to an accumulation of hypoxanthine. Increased intracellular calcium enhances the conversion of xanthine dehydrogenase to xanthine oxidase with, upon reperfusion and reintroduction of oxygen, may produce superoxide and xanthine from the accumulated hypoxanthine and restored oxygen. Further events that trigger reperfusion injury are the activation of sodium-hydrogen exchanger and augmentation of ischemia induced cellular and mitochondrial Ca^{2+} overload, increased osmotic gradient and cell swelling induced by the accumulation of products of anaerobic metabolism, opening of the mitochondrial permeability transition pore (mPTP), resulting in influx of otherwise impermeable proteins, mitochondrial swelling, uncoupling of oxidative phosphorylation and release of pro-apoptotic molecules like cytochrome C (Cyt C) into the cytosol.

The clinical consequences of these events, occurring within minutes of the onset of reperfusion are manifested by myocardial stunning or hypercontracture, infarction, reperfusion arrhythmias, endothelial dysfunction and cell death via necrosis and apoptosis.

1.1.1. Apoptosis in ischaemic/reperfusion injury

Necrosis and apoptosis are two forms of cell death in the myocardium that have been associated with ischemia and reperfusion. Although it has been well documented that necrosis, as a major form of myocyte cell death, rapidly leads to a destruction of a large group of cells after myocardial ischemia and reperfusion, the induction of apoptosis in myocardium, primarily triggered during reperfusion, may independently contribute to the extension of cell death in a dynamic manner. Cell–cell interactions between blood cells and vascular endothelial cells and the release of cytokines and generation of reactive oxygen species from activated neutrophils, endothelial cells and myocytes during reperfusion have been proposed as triggers in the induction of apoptosis. These interactions are initiated within the early movements of reperfusion, and may continue during the ensuing hours and days. Apoptosis depends on the activation of the caspases, a family of proteinases with essential active-site cysteine residues.

Mitogen-activated protein kinases also known as MAP kinases are serine/threonine/tyrosine-specific protein kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as

mitogens, osmotic stress, heat shock or environmental stress and proinflammatory cytokines. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis - among many others. There are three major MAPK in mammalian cells named extracellular signal-regulated kinase-p44/42 (ERK), p38 MAPK (p38) and c-Jun N-terminal kinase (JNK). In the case of classical MAP kinases, the activation loop contains a characteristic TxY (threonine-x-tyrosine) motif that needs to be phosphorylated on both the threonine and the tyrosine residues in order to lock the kinase domain in a catalytically competent conformation. MAPK signaling cascades are organized hierarchically into three-tiered modules. The activation loop phosphorylation is performed by members of the Ste7 protein kinase family, also known as MAP2 kinases. MAP2 kinases in turn, are also activated by phosphorylation, by a number of different upstream serine-threonine kinases (MAP3 kinases). Because MAP2 kinases display very little activity on substrates other than their cognate MAPK, classical MAPK pathways form multi-tiered, but relatively linear pathways. These pathways can effectively convey stimuli from the cell membrane (where many MAP3Ks are activated) to the nucleus (where only MAPKs may enter) or to many other subcellular targets. Although the MAPK activiting stimuli could be proapoptotic or antiapoptotic, the biological outcome of MAPK activation is highly divergent and appears to be largely dependent on the cell type, the environmental stress and the type of MAPK. ERK is an antiapoptotic MAPK which means that its pathways are usually linked to growth factor action and are associated with cellular differentiation, proliferation and tends to promote cell survival. In contrast JNK and p38 MAPK are proapoptotic serine and threonine protein kinases that are activated by various stress stimuli and favour cell death. Since both are usually activated, the balance between them is critical in determining cell fate.

PI3K/Akt pathway may also be important. IP3 is derived from phosphorylated forms of the membrane phospholipid phosphatidylinositol. These phosphorylated forms, generated by phosphoinositide-3-kinases (PI3K) that phosphorylate and activate Akt/PKB (Protein kinase B).[1] Akt regulates multiple biological processes including cell survival, proliferation, growth, and glycogen metabolism. Various growth factors, hormones, and cytokines activate Akt by binding their cognate receptor tyrosine kinase (RTK). Akt binds PIP3 through its pleckstrin homology (PH) domain, resulting in translocation of Akt to the membrane. Akt is activated through a dual phosphorylation mechanism. PTEN, a lipid phosphatase that catalyzes the dephosphorylation of PIP3, is a major negative regulator of Akt signaling. Loss of PTEN function has been implicated in many human cancers. GSK-3 is a primary target of Akt and

inhibitory phosphorylation of GSK-3α or GSK-3β has numerous cellular effects such as promoting glycogen metabolism, cell cycle progression, regulation of wnt signaling, and formation of neurofibrillary tangles in Alzheimers disease. Akt promotes cell survival directly by its ability to phosphorylate and inactivate several pro-apoptotic targets, including Bad, Bim, Bax, and the forkhead (FoxO1/3a) transcription factors. Akt also plays an important role in metabolism and insulin signaling.

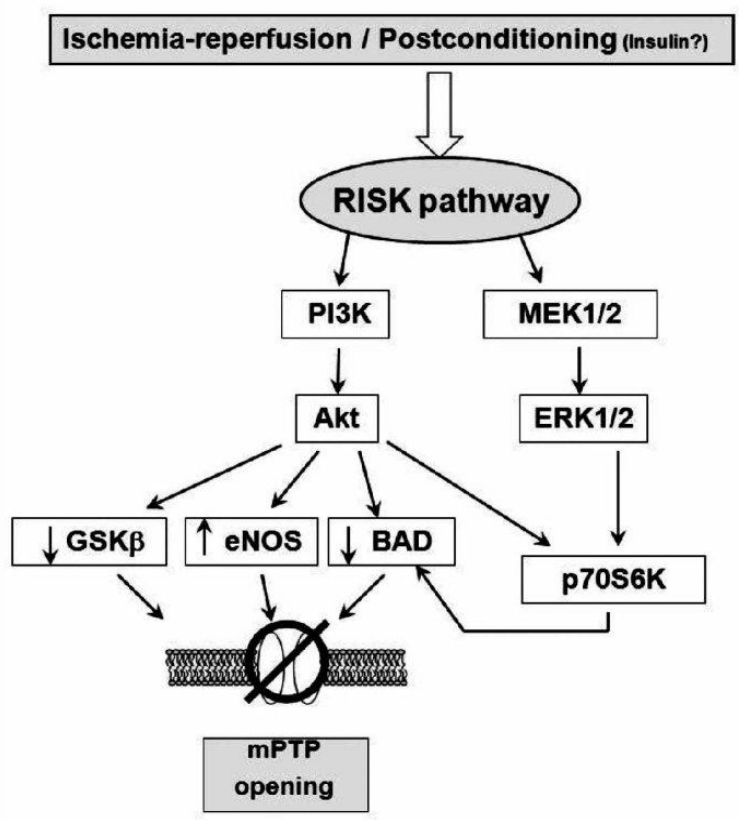
The Reperfusion Injury Salvage Kinase (RISK) pathway refers to a group of innate pro-survival kinases, that include PI3K-Akt, ERK/p42-44 and GSK-3β, which confer powerful cardioprotection on activation at the onset of myocardial reperfusion. It have demonstrated that the activation of these pro-survival kinases reduces myocardial infarct size in the region of 40-50%, furthermore this pathway is also activated by the endogenous cardioprotective phenomena of ischemic pre- and postconditioning.[23]

Glycogen synthase kinase-3 (GSK3) is one of the most important downstream target of the RISK pathway. It is a proline-directed serine-threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase. GSK-3 is found in the cytosol, mitochondria, and nucleus of cells.[4] GSK-3 activity has been associated with many cell processes, including the regulation of multiple transcription factors, nuclear factor κB, endoplasmic reticulum (ER) stress, embryogenesis, apoptosis and cell survival, cell cycle progression, cell migration. Two isoforms, alpha and beta, show a high degree of amino acid homology.[5] Ischemia/reperfusion generates multiple factors that facilitate mPTP opening and it means the first step to the cell death.[6] Phosphorylation of GSK-3β causes the inhibition of the enzyme itself. Phosphorylated GSK-3β inhibits mPTP opening presumably by multiple mechanisms and as a consequence it inhibits the release of cytochrome C from mitochondria, a mechanism preventing apoptotic cell death.[7]

Bcl-2-associated death promoter (Bad) is the other important downstream target of the RISK pathway. Bad protein is a pro-apoptotic member of the Bcl-2 gene family which is involved in initiating apoptosis.[8]. It does not contain a C-terminal transmembrane domain for outer mitochondrial membrane and nuclear envelope targeting, unlike most other members of the Bcl-2 family.[9] After activation, it is able to form a heterodimer with anti-apoptotic proteins and prevent them from stopping apoptosis. Bax/Bak are believed to initiate apoptosis by forming a pore in the mitochondrial outer membrane that allows cytochrome c to escape into the cytoplasm and activate the pro-apoptotic caspase cascade. The anti-apoptotic Bcl-2 and

Bcl-xL proteins inhibit cytochrome c release through the mitochondrial pore and also inhibit activation of the cytoplasmic caspase cascade by cytochrome c.[10]

Dephosphorylated Bad forms a heterodimer with Bcl-2 and Bcl-xL, inactivating them and thus allowing Bax/Bak-triggered apoptosis. When Bad is phosphorylated by Akt/protein kinase B (triggered by PIP3), it forms the Bad protein homodimer. This leaves Bcl-2 free to inhibit Bax-triggered apoptosis.[11] Bad phosphorylation is thus anti-apoptotic, and Bad dephosphorylation (e.g., by Ca^{2+}-stimulated Calcineurin) is pro-apoptotic.



Pastor Luna-Ortiz, Juan Carlos Torres, Gustavo Pastelin, Arch Cardiol Mex 2011;81(1):33-46

Figure 1. The mechanism of protection induced by ischemia reperfusion (that could also be triggered by ischemic postconditioning)

In our study we focused on the activation of members of MAPK and RISK families: p38, ERK, GSK-3β protein kinase and Bad.

1.2. ISCHAEMIC POSTCONDITIONING

The procedure called myocardial postconditioning, when the heart can be protected against the ischemia-reperfusion injury with brief coronary occlusions performed just at the beginning of the reperfusion. Postconditioning is controlled reperfusion where the beneficial outcomes observed with include reduction in infarct size[12], in endothelial dysfunction, in neutrophil adherence, and in apoptosis.[13] Postconditioning was first described by Zhao and colleagues in dogs, in which it reduced the myocardial injury to an extent comparable to ischemic preconditioning.[14]

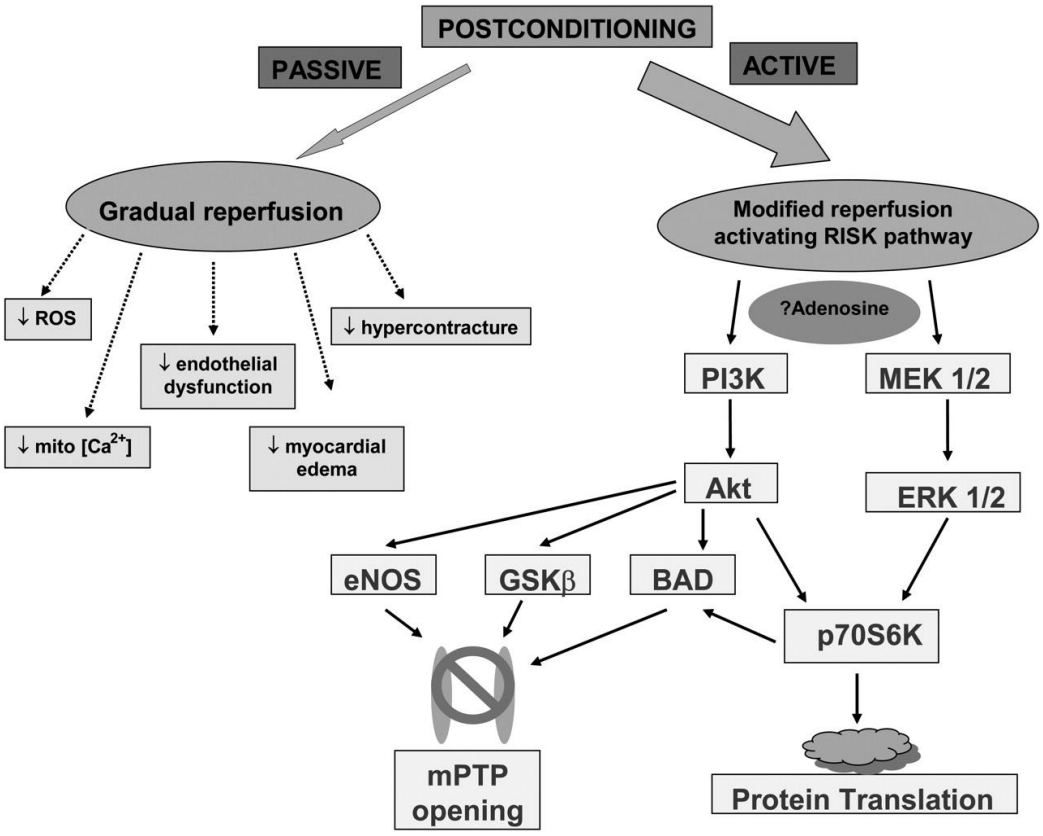
1.2.1 The proposed mechanism of protection induced by ischaemic postconditioning

Studies have documented that several pathways and molecular components are involved in the cardioprotective effects of postconditioning. Any cardioprotective strategy applied at the time of reperfusion must provide protection against the known mediators of lethal reperfusion injury, which include sudden burst of free radicals, mitochondrial calcium overload, and increased endothelial dysfunction and reduced nitric oxide production. However, there is not a scheme that could help to understand the role of each described component and the final or direct effectors of postconditioning (PC). The mechanisms of PC are realised on passive and active way. The damaging effects of reperfusion are due to the many biochemical and physical perturbations that occur in the transition from ischemia to reperfusion. Immediate full-flow reperfusion leads to pressure overload and resultant myofibrillar stretching. This ultimately leads to myocardial edema, which is aggravated by microvascular injury, hypercontracture, and myocyte death.

According to the passive way in the course of reperfusion PC hang up the washout of adenosine, decreases extracellular levels and the accumulation of noxious metabolites which decrease 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 enhances nitric oxide release by endothelial cells, which further attenuates superoxide anion levels and both neutrophil activation and adherence to the endothelial cells. PC reduces the intracellular buildup of oxidants and calcium in cardiomyocytes, which achieving the inhibition of mPTP opening thereby inhibiting both apoptosis and necrosis. in this way.

As described, mitochondria and mPTP opening have been proposed to play an essential role in reperfusion injury and, regarding the active way during the early stages of reperfusion, there is an up-regulation of pro-survival kinases termed: the RISK pathway, which, recently, has promoted a renewed interest in cardioprotective reperfusion strategies. As it was early mentioned this pathway comprises to PI3-kinases and ERK, which could be activated by ischemia-reperfusion injury or by PC or pharmacological PC. The PI3-kinases are a family of enzymes involved in several functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. These enzymes are capable of phosphorylating the 3-position hydroxyl group of the inositol ring of phosphatidylinositol. Particularly, the PI3K-Akt and MEK kinases (MEK kinase = MAPK/ERK kinase; mitogen-activated protein kinase/extracellular signal-regulated kinase) have shown to be important components of the cell survival pathway and have antiapoptotic effects. There are several potential mechanisms, through which this pathway mediates inhibition of mPTP opening. These are: 1) phosphorylating and inhibiting GSK3β; 2) phosphorylating eNOS and producing nitric oxide, which has been demonstrated to inhibit mPTP opening; and 3) phosphorylating Bad, either directly or indirectly via p70S6 kinase, thereby the protective effects of ischemic PC are abolished. Latterly, Davidsson and colleagues [15] revealed that insulin-mediated phosphorylation of PI3K-Akt protects the myocyte against oxidative stress by inhibiting mPTP. This suggests that the pharmacological activation of the RISK pathway by insulin at the onset of reperfusion protects against ischemia-reperfusion injury by reducing the probability that the mPTP will open. This mechanism could take part in the cardioprotective effect of "GIK" solution, which is composed by insulin in combination with glucose and potassium.

However, ischemic PC protect the heart through the inhibition of mitochondrial permeability transition pore opening. Taken together PC is likely to represent a form of modified reperfusion that paradoxically has been known for a number of years to be beneficial to the ischemic myocardium.



Andrew Tsang , Derek J. Hausenloy , Derek M. Yellon, American Journal of Physiology - Heart and Circulatory PhysiologyPublished 1 July 2005

Figure 2. Hypothetical scheme of the possible mechanisms of protection induced by ischaemic postconditioning are realised on passive and active way.

1.3 CORONARY ARTERY BYPASS GRAFTING AND PERIOPERATIVE MYOCARDIAL INFARCTION

Over the last decade, elective coronary artery bypass grafting (CABG) is the main revascularization modality in the treatment of the patients with multivessel coronary artery disease (CAD) and has progressed to a very standardized and safe surgical procedure with low mortality and low rates of myocardial, neurologic, renal and other adverse events. Perioperative myocardial infarction (PMI) is the most common cause of morbidity and mortality, it means a serious complication following CABG surgery with an incidence between 3 and 30%. MI following CABG was associated with a significant increase in

intensive care unit time, hospital length of stay, and overall costs, which contributed to greater hospital and physician service costs.[16] Predictive risk factors and the incidence of graft closure in this setting have not been clearly established.

The diagnosis of classical clinical symptoms of myocardial ischaemia is a clinical challenge. Surgeons rely upon electrocardiogram (ECG) modifications (new ST segment alterations or new Q wave), obstinate malignant arrhythmias, elevation of cardiac biomarkers, persistent low cardiac output syndrome (LCOS) and new echocardiography wall motion abnormalities to identify possible myocardial injury.[17] Myocardial damage following CABG surgery is due to two different causes classified as graft or non-graft related.[18] The incidence of early graft dysfunction is ~3%. Non-graft-related ischaemia is related to inappropriate myocardial protection, excessive surgical manipulations, intraoperative defibrillation, air or plaque embolization or genetic alterations.[19] Graft-related injury is associated with: early graft thrombosis, anastomotic stenosis, bypass kinks, overstretching or tension, significant spasm or incomplete revascularization. Hence, discrimination between graft-related ischaemic events from other reasons must be made thoroughly.

The PREVENT IV study identified such intraoperative risk factors for PMI as prolonged cardiopulmonary bypass or aortic cross-clamp times, perioperative myocardial ischemia, and inadequate revascularization. Other well-established risk factors for PMI included age, left main coronary artery disease and three-vessel disease, impaired left ventricular function, unstable angina, recent MI, and emergent operations.[20]

Early reintervention has been proposed to allow myocardial rescue to preserve ventricular function after CABG surgery since PMI is associated with congestive heart failure and significant adverse outcomes.

Because it is difficult to determine the severity of perioperative myocardial damage after CABG have lead to use of different diagnostic markers, which in turn has contributed to a wide variation in the reported incidence and clinical concequences.

1.4 GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE

1.4.1 Glutathione

Glutathione (GSH) is one of the dominant low-molecular-weight thiol (0.5–10 mmol/L in cells) known to play an important role in defense mechanisms. GSH functions as a substrate in antioxidative defense mechanisms by conjugating to toxic electrophilic compounds,

scavenging free radicals, and reducing peroxides. GSH is associated with stress resistance owing to its redox-active thiol group, and it is an important antioxidant responsible for the maintenance of antioxidative machinery of the cells under stress. Several important catalytic enzymes that utilize GSH in defense mechanisms, such as GST and glutathione reductase (GR) show differential patterns of activity in tissues exposed to oxidative stress conditions.

85–90% of the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes)[21]. In most tissues, GSH is predominantly present in its reduced form, its extent in the extracellular concentrations is relatively low (e.g., 2–20 μmol/L in plasma)[2223].

Following the explanation of its chemical structure, thinking of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). The regeneration of GSH from oxidized glutathione (GSSG) is catalyzed by GR enzyme. GSH is oxidized by various free radicals and oxidants to glutathione disulfide (GSSG), while GR uses NADPH to reduce GSSG to GSH.

R-O-OH+GSH→R-OH+GSH-OH

GSH-OH+GSH→GS-SG

Reduction of an organic hydroperoxide to the monohydroxy alcohol by GSH conjugation

Most reactive oxygen species are reduced, directly by GSH; and by the activity of GR, GSH scavenges hydrogen peroxide which is involved in the detoxification of lipid peroxides rather than hydrogen peroxide per se.

The [GSH]:[GSSG] ratio is often used as an indicator of the cellular redox state. GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, its value is >10 under normal physiological conditions.

The rate of this couple can be affected by other redox couples, including NADPH/NADP+ and thioredoxinred/thioredoxinox. It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including Akt, calcineurin, nuclear factor κB, JNK, apoptosis signal-regulated kinase 1, and MAPK), thereby reducing

cell proliferation and increasing apoptosis.[24] A major factor that affects GSH homeostasis is its utilization by conjugation, primarily via GST[2526].

1.4.2 Glutathione S-transferase

Many defense strategies have evolved in human body allowing it to deal with the threat of a broad spectrum of both foreign and endogenous cytotoxic and genotoxic compounds. Phase II enzymes deactivate reactive chemicals by appending a hydrophilic moiety (e.g. glutathionyl, glucuronyl, or sulphuryl) to the functional group. The GST superfamily represent an essential part of the phase II detoxification mechanism. These intracellular proteins are found in most aerobic eucaryotes and procaryotes, and by catalyzing the S-conjugation between the thiol group of GSH and an electrophilic moiety in the hydrophobic and toxic substrate, they protect cells against chemical induced toxicity and stress.[2728]

The GSTs catalyze the general reaction shown below:

$GSH + R\text{-}X \rightarrow R\text{-}SG + HX$

The important noncatalytic functions of the GSTs include their capacity to sequester carcinogens, their involvement in the intracellular transport of a wide spectrum of hydrophobic ligands, and their modulation of signaling pathways.

The mammalian cytosolic GSTs are divided into seven classes and are designated by the names of the Greek letters, including alpha (α), mu (μ), sigma (σ), theta (θ), pi (π), omega (ω), and zeta (ζ)[29 30 31 32 33]. The differencities of the classes are based on amino acid sequence comparisons, substrate specificities, sensitivities to inhibitors, N-terminal amino acid sequences, exon-intron comparisons, immunological and antibody cross-reactivities. These GSTs can be divided into three major families of proteins: cytosolic, mitochondrial, and microsomal, of which the cytosolic GSTs represents the largest family.[34] The activity of the encoded proteins is needed under many different conditions. Each class is encoded by a single gene or a gene family. The genes encoding the GSTs are polymorphic. It has been suspected that these polymorphisms are functionally significant and that the frequencies of their allelic variants differ among human populations. The gene expression of at least a subset of GST genes is activated by the occurrence of oxidative stress, and the activity of the encoded proteins is needed to protect cells against oxidative damage. The GST is able to change level of cellular GSH in response to production of ROS has been involved in protection of cells from ROS-inducing agents.[3536] Accumulation of ROS in response to myocardial ischemia

results in the activation of multiple stress kinase cascades, including the ASK1 (apoptosis signal-regulating kinase 1), MEKK1 (mitogen-activated protein kinase kinase 1), MAPK, namely JNK, p38 MAPK, ERK, Akt/PKB and GSK-3β protein kinase regulated signaling pathways.[373839] The balance between different stress signaling cascades seems to be among the key determinants in dictating the cell's fate, although the mechanisms are not totally understood.[404142] More recently, the different isoenzymes of GST classes have been shown to relate with members of the MAPK pathways involved in cell survival and death signaling. This is a non-enzymatic role in which GSTs function to sequester the kinase in a complex, thus preventing it from acting on downstream targets. The result of this action is a regulation of pathways that control stress response to ischaemia/reperfusion (I/R) injury, cell proliferation and apoptotic cell death.[43]

1.4.2.1 Glutathione S-transferase PI (GSTP1) (GSTP)

Laisney et al. (1984) stated that GSTP1 is present in all tissues and cells, with the exception of red cells, moreover in leukocytes, only GSTP1 is found.[44] Morrow et al. was the first, who reported that the GST-pi gene includes 7 exons and spans approximately 2.8 kb.[45] In the same year Moscow et al. and Board et al. mapped the GST-pi gene to 11q13 using in situ hybridization.[46] [47]

Examining the enzyme Bora et al. identified GST pi as fatty acid ethyl ester synthase III (FAEES3), a heart enzyme that metabolizes ethanol nonoxidatively.[48]

Ali-Osman et al. (1997) isolated cDNAs corresponding to 3 polymorphic GSTP1 alleles, GSTP1*A, GSTP1*B, and GSTP1*C, expressed in normal cells and malignant gliomas. The variant cDNAs result from A-to-G and C-to-T transitions at nucleotides 313 and 341, respectively. The transitions changed codon 105 from ATC (ile) in GSTP1*A to GTC (val) in GSTP1*B and GSTP1*C, and changed codon 114 from GCG (ala) to GTG (val) in GSTP1*C. Both amino acid changes are in the electrophile-binding active site of the GST-pi peptide. The alleles are expressed in various homozygous and heterozygous combination. Individuals who are heterozygous for this genetic polymorphism have different enzymatic activity. Polymorphism altering or reducing these enzyme detoxification activities could increase a person's susceptibility to diseases including CAD. The variant GSTP1 allele may increase susceptibility due to decreased detoxifying potential. It is proved that deleted polymorphisms in the GST genes may also influence the susceptibility to CAD by modulating the detoxification of genotoxic atherogens.[49] In a most recent research GSTP1 effect was examined in a rat myocardial infarction-induced model by Andrukhova et al. GSTP1 mRNA

and protein expression were elevated in failing myocardium. Heart failure was associated with higher active JNK and p38 protein expression but reduced GSTP1 binding activity to JNK and p38. It has been proven that GSTP1 inhibits JNK and p38. Single-dose GSTP1 treatment reduced infarct area, apoptosis, and the expression of JNK, p38, nuclear factor κB, and proinflammatory cytokines and improved thinning ratio, cardiac index and output, stroke volume, ejection fraction, regional wall motion, and survival compared with control. GSTP1 activation early after myocardial infarction (AMI) results in long-term beneficial structural and functional effects that prevent progression to heart failure. According to the authors GSTP1 could be a novel adjunct myocardial salvage approach in patients after AMI.[50]

Menegon et al. stated the hypothesis that Parkinson disease is secondary to the presence of neurotoxins and that pesticides are possible causative agents. Because enzymes of glutathione transferase metabolize xenobiotics, including pesticides, they investigated the role of GST polymorphisms in the pathogenesis of idiopathic Parkinson disease. Menegon et al. demonstrated that GSTP1 expressed in the blood-brain barrier thereby may influence response to neurotoxins and explain the susceptibility of to the parkinsonism-inducing effects of pesticides.[51] Wilk et al. also proved evidence suggesting that exposure to herbicides may be an effect modifier of the relationship between GSTP1 polymorphisms and age of onset in Parkinson disease.[52]

Gilliland et al. noted that GSTM1 and GSTP1 modify the adjuvant effect of diesel exhaust particles on allergic inflammation. They exposed ragweed-sensitive patients intranasally with allergen alone and with allergen plus diesel exhaust particles, and found that individuals with GSTM1 null or GSTP1 ile105 wildtype genotypes showed significant increases in IgE and histamine after inhalation with diesel exhaust particles and allergens; the increase was largest in patients with both the GSTM1 null and GSTP1 ile/ile genotypes.[53]

The same allele pair polymorphism and the treatment response of the platinum-based chemotherapy in non-small cell lung cancer patients were analysed in a meta-analysis study where it was found that the GSTP1 A313G and GSTM1 null/present polymorphisms could predict the treatment response of the platinum-based chemotherapy in lung cancer patients.[54]

1.4.2.2 Glutathione S-transferase Mu-1 (GSTM1), (GSTM)

Among numerous defence mechanism against oxidative stress and I/R injury, the endogenous antioxidant enzyme GST are crucially involved in cellular response to stress, apoptosis and proliferation.

GST is responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates.

First of all Board et al. (1981) showed that one of the most active GST of liver is the products of autosomal loci GSTM1.[55] Strange et al. (1984) comfirmed that GSTM1 is easily demonstrable in adult liver, kidney, adrenal and stomach but is only weakly expressed in skeletal and cardiac muscle and not at all in fetal liver, fibroblasts, erythrocytes, lymphocytes and platelets.[56]

By used oligonucleotide primers specific for intron 5 sequences in the GSTM1 gene Zhong et al. amplify a unique 718-bp fragment[57]. They confirmed the designation to 1p by analysis of DNA from a panel of somatic cell hybrids and refined the localization to 1p13 by linkage analysis. One year later it has been used locus-specific polymerase chain reaction (PCR) primer pairs to map 5 glutathione transferase genes to chromosome 1: GSTM1, GSTM2, GSTM3, GSTM4, and GSTM5[58]. For GSTM1, the assignment was confirmed by Southern blot hybridization. The location of the 5 genes on chromosome 1 was confirmed by fluorescence in situ hybridization and regionalized to a point in or near 1p13.3. Xu et al. discovered that 4 mu GST genes are tightly clustered. These genes are located approximately 20 kb separately and are arranged in the following sequence: 5-prime--GSTM4--GSTM2--GSTM1--GSTM5--3-prime[59]. They edited a partial physical map of the GST gene cluster on 1p13.3 and identified the end points of the GSTM1 deletion. The same deletion was found in all unrelated individuals examined.

Data on gene frequencies of allelic variants were relatively early discovered. A null allele at the GSTM1 locus has a high frequency of about 0.7, however the null phenotype has a frequency of greater than 50% among Caucasian, Chinese, and Indian populations. [60] [61] In an other study Cotton et al. reported that the frequency of the GSTM1 null genotype ranges from 23 to 62% in different populations around the world and is approximately 50% in Caucasians.[62] The GSTM1 null phenotype appears to be caused by a partial gene deletion. It is associated with complete absence of GSTM1 enzyme activity.

The GSTM1 null allele may result from unequal crossing-over. McLellan et al. found patients with ultrarapid GSTM1 enzyme activity were heterozygous for a tandem GSTM1 gene duplication. Probably the duplication was generated as the reciprocal product of the homologous unequal crossing-over event that produces the null allele.[63]

In the year 2009 through a genomewide association study, Huang et al. identified a significant association between rs366631, approximately 11 kb downstream of the GSTM1 gene, and GSTM1 expression. The authors determined that the rs366631 single nucleotide

polymorphism (SNP) is a nonpolymorphic site. The authors concluded that rs366631 is a pseudo-SNP that can be used as a GSTM1 deletion marker.[64]

Lack of GSTM1 can be a risk factor for cancer by increasing sensitivity to particular chemical carcinogens.[65] [66] As early as 1987 has been published that the GSTM1 null allele is more frequent in livers with hepatitis and carcinoma than in controls.[67] Six years earlier Board supported the notion that individuals with the null allele are exposed to elevated levels of certain electrophilic carcinogens.

The GSTM1 null polymorphism was a significant predictor of global gene expression in acute lymphoblastic leukemia, dividing patients based on their germline genotypes. Although GSTM1 expression is concentrated in liver, it is involved in the conjugation (and thus transport, excretion, and lipophilicity) of a broad range of endobiotics and xenobiotics, which French et al. (2005) suggested could plausibly have consequences for gene expression in different tissues.[68]

Besides these it was found an association between the GSTM1 null phenotype and susceptibility to lung cancer[6970] and colorectal cancer[71]. Head and neck cancer showed also strong association with homozygosity for a null allele of the GSTM1 gene.[72]

Carless et al. examined the role of genetic polymorphisms in susceptibility to solar keratoses development.[73] A significant association between GSTM1 genotypes and solar keratoses development was detected, with null individuals having an approximate 2-fold increase in risk for solar keratoses development and a significantly higher increase in risk in conjunction with high outdoor exposure.

The formation of DNA and protein adducts by environmental pollutants is modulated by host polymorphisms in genes that encode metabolizing enzymes. The results showed the combined effects of genetic polymorphisms and indicated that, due to the complex carcinogen exposure, simultaneous assessment of multiple genotypes may identify individuals at higher cancer risk.

Contradictory articles also can be found in the literature. Isothiocyanates which have been shown in animals to have strong chemopreventative properties against lung cancer are thought to be eliminated by GST enzymes, most notably GSTM1 and GSTT1. Both GSTM1 and GSTT1 genes have null alleles with homozygous null phenotypes, resulting in no enzyme being produced. Individuals who are homozygous for the inactive form of either or both genes probably have higher isothiocyanate concentrations because of their reduced elimination capacity. Furthermore, and implicit in the mendelian randomization approach, the roles of GSTM1 and GSTT1 genes are likely to be independent of other dietary or lifestyle factors. To investigate the role of cruciferous vegetable consumption in the prevention of lung cancer in

interaction with GST genotypes, Brennan et al. (2005) investigated this relation in a case-control study of 2,141 cases and 2,168 controls in 6 countries of central and eastern Europe, a region that has traditionally high rates of cruciferous vegetable consumption. Weekly consumption of cruciferous vegetables protected against lung cancer in those who were GSTM1-null, GSTT1-null, or both. No protective effect was seen in people who were both GSTM1- and GSTT1-positive.[74]

1.4.2.3 Glutathione S-transferase Theta-1 (GSTT1)(GSTT)

For a long time the GSTs of the theta class were largely overlooked until Pemble et al. (1994) reported the cDNA cloning of a human theta-class GST, termed GSTT1.[75] The deduced 239-amino acid GSTT1 protein shares 80% sequence identity with the rat homolog. By in situ hybridization studies, Webb et al. (1996) mapped the GSTT1 gene to 22q11.23 position.[76]
GSTT1 has been implicated in detoxifying mutagenic electrophilic compounds.
According to research by Pemble it is proved that the glutathione-dependent conjugation of halomethane is polymorphic in humans, with 60% of the population classed as conjugators and 40% as nonconjugators. They showed that the GSTT1 gene was absent from 38% of the population by PCR and Southern blot analyses. The presence or absence of the gene was coincident with the conjugator (GSTT1+) and nonconjugator (GSTT1-) phenotypes, respectively. In humans, the GSTT1 enzyme is found in the erythrocyte and this may act as a detoxification pool.
Individual differences in the metabolism of methyl bromide, ethylene oxide, and methylene chloride in human blood have been ascribed to the genetic polymorphism of GSTT1 (Peter et al., 1989; Pemble et al., 1994).[77] As regards the GSTT1 enzyme activity measured in erythrocytes, an individual can be divided 3 groups: nonconjugators, low conjugators, and high conjugators (Hallier et al., 1990)[78]. In an in vitro study Schroder et al found that genotoxic effects such as induction of sister chromatid exchanges (SCE) after exposure of human blood to methyl bromide and other agents were present to be more higher in nonconjugators. [79](Schroder et al., 1995). Therefore it is seems clear that the nonconjugator phenotype is a result of the homozygous absence of a GSTT1 allele (GSTT1*0). This allele represents a partial or complete deletion at the GSTT1 gene locus.
Several other studies have shown a difference in susceptibility toward toxic effects in nonconjugators and conjugators.

Thus, Pemble et al. (1994) stated that characterization of the GSTT1 polymorphism would enable a more accurate assessment of human health risk from synthetic halomethanes and other industrial chemicals.

In an other study Wang et al. [80]investigated whether the association between maternal cigarette smoking as the single largest modifiable risk factor for intrauterine growth restriction and infant birth weight differs by polymorphism of GSTT1. For the GSTT1 genotype, they found the estimated reduction in average birth weight was 285 g and 642 g for the present and absent genotype groups.

Patients with reduced ability to metabolize environmental carcinogens or toxins may be at risk of developing aplastic anemia and myelodysplastic syndromes (MDS). Lee and coworkers [81]investigated whether homozygous deletions of GSTM1 and GSTT1 affect the probability of developing aplastic anemia. They realized that the presence of GSTM1 and GSTT1 gene deletions was significantly higher for aplastic anemia patients than for healthy controls. Interestingly all aplastic anemia patients who had chromosomal abnormalities showed GSTT1 gene deletions. Chen et al. [82]compared the frequency of the GSTT1 null genotype in 96 patients with MDS and 201 cancer-free controls of similar age, race, and sex. The frequency of the GSTT1 null genotype was 46% among MDS cases and 16% among controls. Inheritance of the GSTT1 null genotype was calculated to confer a 4.3-fold increased risk of MDS.

Two another study further confirmed the above mentioned statements. Wiebel et al. (1999) analyzed 29 persons in 3 generations of a large family; phenotyping and genotyping the point of view of GSTT1 was performed. The enzyme activity of high conjugators was twice as high as that of low conjugators. This distribution of GSTT1 phenotypes clearly showed a mendelian intermediary inheritance, where the gene-dosage effect results in a doubled enzyme expression in the presence of 2 functional GSTT1 alleles.[83]

Chen et al. [84]described the genotypes of GSTM1 and GSTT1 in whites and blacks. The frequency of the null genotype for GSTM1 was higher in whites and that for GSTT1 null was higher in blacks. The 'double null' genotype frequency was not significantly different so they stated that the 2 polymorphisms are independent and may differ by race and different populations.

2. AIMS AND HYPOTHESIS

In the first part our aim was to identify the biological role of GST and assess the effect of GST inhibition (using its potent inhibitor, ethacrynic acid [EA]) by using an in vivo myocardiac infarction model on rat.

- Firstly it was targeted to investigate the effect of GST inhibition on myocardial tissue damage and oxidative stress parameters.

- It was aimed to observe the alteration of proteins, MAP kinase and RISK pathways and finally cardiomyocyte apoptosis in case of occurrence of myocardial infarction.

- We targeted to investigate the effect of inhibited GST to the favorable effects of ischaemic postconditioning.

In the second part of our investigations we aimed to determine the existence of association between the genetic polymorphisms of metabolizing genes GSTP1, GSTM1, and GSTT1 and the presence of perioperative acute myocardial infarction in a cohort of patients undergoing cardiac surgery with cardiopulmonary bypass.
The variant GST alleles may increase susceptibility due to decreased detoxifying potential. Thus assumed that the differences of activity in the detoxification enzyme GST is associated with cardiovascular disease (PMI).

3. THE ROLE OF GLUTATHIONE S-TRANFERASE IN AN IN VIVO ACUTE MYOCARDIAL INFARCTION RAT MODEL

3.1 INTRODUCTION

It has been well investigated that oxidative stress is a major apoptotic stimulus in the cardiac diseases. Apoptosis can be caused by both prolonged ischaemia/hypoxia and by reperfusion. The mechanisms of reperfusion-induced cell death are not completely understood, but it seems that the occurrence of oxidative stress related to the generation of ROS may play an important role.

The PC is derived from the concept of partial or controlled reperfusion[85]. It has been reported that intermittent reperfusion, which equates to the current concept of ischemic PC, abolished fibrillation[86 87] , then a few years later found that PC was as effective as preconditioning in preventing ventricular fibrillation in cats[88]. When Z.Q Zhao and colleagues published their first study on ischemic PC in a myocardial ischemic model have PC studies thrived[89]. The protective effects of PC in myocardial ischemia has been confirmed by many other studies[9091] as well as in vitro settings[92]. Taken together, the concept of ischemic PC in myocardial research has been well-established. PC is induced immediately or a few minutes after reperfusion, and it is the main form of PC in heart research.

The GSTs are one of the key groups of detoxification enzymes. The EA is a commonly used diuretic drug and in addition a potent inhibitor of GST enzymes. EA itself inhibits GST through reversible covalent interactions, on the other hand nonenzymatic GSH conjugation of EA also exist.

Two major MAPKs, namely p38 and ERK are activated in response to a wide variety of stimuli including growth factors, G protein-coupled receptors, and environmental stresses thus play a pivotal role in the transmission of signals from cell surface receptors to the nucleus [9394]. The pathways regulated by p38 contribute importantly to apoptosis. The mechanisms by which p38 induce apoptosis are largely cell and stimulus specific. ERK activation are protective against apoptotic cell death. [959697].

The RISK pathway is a family of protein kinases that appear to be activated during reperfusion and to confer cardioprotection by preventing myocyte calcium overload,

inhibiting MPTP opening, and recruiting antiapoptotic pathways[9899] such as the phosphorylation and inhibition of the proapoptotic proteins BAX and Bad, the inhibition of caspase 3 activation, and the phosphorylation and activation of p70s6K (which acts to inhibit Bad[100]) and the phosphorylation, activation of the antiapoptotic protein Bcl-2[101] and inhibition of GSK -3β.

For the better understanding of the processes in an in vivo experimental animal model the occlusion phenomenon can be mimicked by ligation of one of the coronary arteries. The rat model of myocardial infarction by coronary artery occlusion was first published in 1954.[102]

In present study we aimed to investigate the biological role of GST in heart under acute myocardial I/R. Because GST activity have adaptive response to oxidative stress in the heart, thus we hypothesized that GST inhibition via administration of EA might aggravate the severity and outcome of AMI. It was also aimed to determinate the effect of GST inhibition on postcondition. Furthermore it was hypotetised that alterations in MAPK (p38, GSK-3β and ERK) and Bcl-2 gene family (Bad), GST activity caused by inhibition of GST cause altered iscemic tolerance and postcondition in myocardium.

3.2 MATERIALS AND METHODS

3.2.1. Animal model

70 male Wistar rats, weighing between 200-250 g were used in the present study from Charles River Breeding Laboratories (Hungary, Isaszeg). The animals were placed 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 (BA02/2000-9/2008).

3.2.2 Surgical preparation

The animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (500 mg pro ampul) and diazepam (10 mg pro ampul). The ratio was 1:1 (0,2 ml / 100 g) and the animals were placed on a heating pad. The skin was disinfected and fine isolation a tracheotomy was performed. The lungs were ventilated (Sulla 808, Drager , Lübeck, Germany) at a frequency of 60-65 breaths/min and a tidal volume of 3-4 ml. ECG was placed and the carotid artery was catheterized (22 gauge) for blood pressure measurement (Siemens Siracust 1260, Düsseldorf, Germany). The chest was opened by midline sternotomy. A 5-0 prolene (Ethicon 5/0, 1-metric, TF) ligature was passed around the left anterior descending (LAD) coronary artery and through a snare. In general the site of vessel encirclement was on the long axis of the left ventricle towards the apex approximately one-fourth of the distance from the atrioventricular groove to the left ventricular apex. Temperature was measured inside the pericardial cradle (Siemens Sirem, Digital Thermometer, Düsseldorf, Germany) and maintained between 38,3˚C and 38,7˚C by adjusting a heating pad. The chest was then closed and the wound was covered with warm, wet compress to minimize heat and fluid losses. The ligature was after 40 minutes removed and the LAD was reperfused for 120 minutes. The vena cava preparation was performed for collecting blood samples.

3.2.3. Effect and administration of ethacrynic acid

Present study used EA for pharmacological inhibition of GST. EA, a diuretic, inhibits GST in two ways. It has been shown to be a substrate of GST enzymes, on the other hand nonenzymatic GSH conjugation of EA also proved. Moreover this EA-GSH complex is an inhibitor of GSTs as well due to its stronger affinity for the enzymes. On the other hand EA itself inhibits GST trough reversible covalent binding.[103]

We recalculated 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 dose of EA was 8,6 mg/kg and the solution was injekted to the animals intraperitoneally 24 hours and 1 hours before the operation.

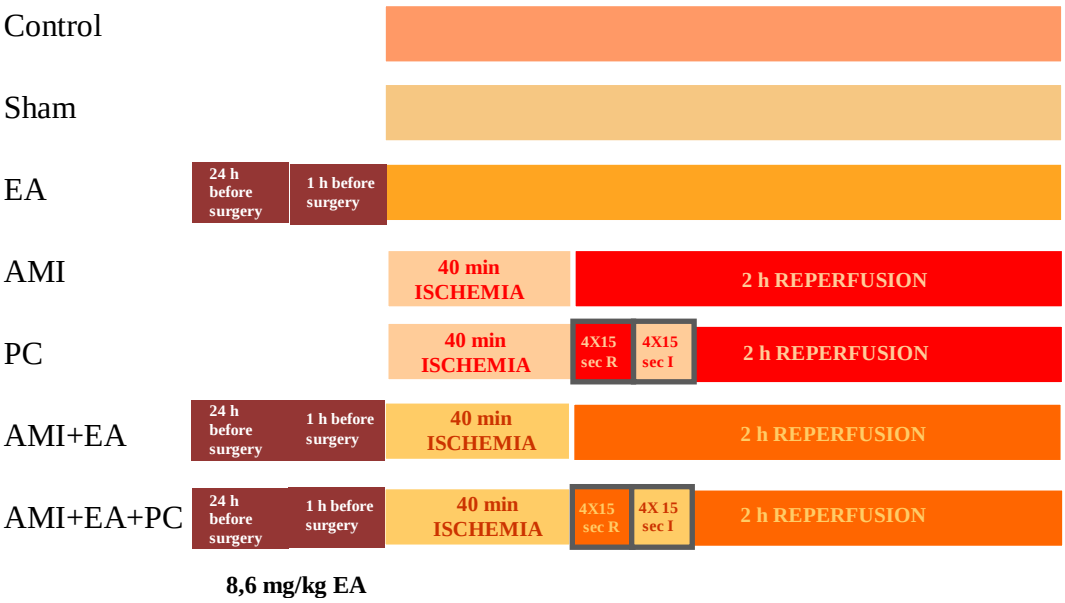
3.2.4. Protocol of ischaemic postconditioning

In the ischaemic postconditioned group and in the ethacrynic acid threated PC group, after the 40 minutes ischaemic phase 15 seconds reperfusion – 15 seconds ischaemic periods were used for 4 times. Myocardial ischaemia was easily noticeable by the emergence of a dusky, bulging region of myocardium (careful note was made of anatomic landmarks of this region). The effectiveness of this intervention was confirmed by the appearance of epicardial cyanosis and by the immediate occurrence of ST-segment elevations in the ECG (Siemens Sirecust 1260, Düsseldorf, Germany). At the end of the 2-min postconditioned period of coronary artery, the suture was released and removed to provide the appropriate reperfusion, which was verified by the disappearance of the ECG changes within 5 min in every animal.

3.2.5. Experimental protocol

In our experiments the animals (70 rats) were divided into 7 groups (10 animals in each group). In control animals (group 1; control) the heart was excised right after thoracotomy. In the sham operated rats (group 2; sham) after the thoracotomy we opened the chest by midline sternotomy for two hours without intervention. Rats in the third group (group 3; EA) were treated with EA as described before. In the fourth group (group 4; AMI) after we closed the LAD for 40 minutes applied 2 hours reperfusion. Next group was the PC group (group 5, PC) as previously discussed. Rats in the sixth group (group 6; AMI+EA) underwent EA treatment combined with LAD occlusion and two hours reperfusion procedure. Finally, in the seventh group (group 7; AMI+EA+PC) the PC was applied following a pretreatment with EA. (Figure 3.)

Figure 3. Experimental protocol of rats



Peripheral blood samples were collected from the animals at the end of the reperfusion phase. The serum samples were harvested and stored at minus 80˚C until biochemical assays. After the experimental period the heart was rapidly excised and rinsed in ice-cold physiological saline. The ischaemic zone was excised on the basis of the previously defined landmarks. The tissue was snap frozen in liquid N2, and stored for not more than 3 days at -82C before Western-blot analysis. Another part of the ischaemic zone was fixed with 10% neutral buffered formalin.

3.2.6. Haemodinamics, arrhytmias and fibrillation

Following a control measurement, ECG, heart rate and systemic blood pressures were monitored and recorded every 10 minutes during the whole procedure. Furthermore occurrence of any ventricular tachycardia and fibrillation were recorded automatically. In case of fibrillations, cardioversion was immediately attempted. Hearts that needed more than 3 consecutive cardioversions to convert ventricular fibrillation were excluded from the study.

3.2.7. Measuring infarct size by the tetrazolium method

Tetrazolium staining has emerged as the most popular method which allows the early detection of myocardial infarction in a whole heart. This technique takes advantage of the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts to form a formazan pigment[104]. Ischemic myocardium is extremely sensitive to temperature. It has been reported that cooling salvages 7% of the risk zone per degree of cooling. Thus allowing a rabbit heart to cool just 2 degrees to 35º C will reduce average infarct size from 35% to 21% in untreated rabbits undergoing a 30 min coronary branch occlusion.[105] Inadequate temperature control is a major source of noise in infarct size measurement. It was very important that the animals were placed on a heating pad, their temperature was maintained between 38,3˚C and 38,7˚C and the wound was covered with warm, wet compress to minimize heat and fluid losses. We use the nitro blue tetrazolium (Sigma catalog # N6876) powder which is diluted in a phosphate buffer. This is a 2 part buffer system consisting of low pH NaH^2PO^4(0.1 M) and a high pH system consisting of Na^2HPO^4(0.1M). It were used about twice as much of the high buffer as the low in the pH 7.4. Administration of tetrazolium salts was 1% weight/volume (1g/100ml). The tissue can be cut more easily when it is in a semi frozen state and the stain is a more reliable discriminator if the tissue has gone through a freeze-thaw cycle. In the case of rat hearts it has been sliced into ~3 mm slices simply by eye. To freeze the tissue it has been putting in a -20ºC freezer for an 1 hour is ideal. Once the tissue is solid it can be sliced. It is important to use food wrap because it keeps the heart from freeze-drying. Freeze-dried tissue will always be tetrazolium negative. The slices are then incubated in the tetrazolium stain at a temperature of 37ºC for 20 minutes. The surviving tissue should turn a dark blue. Once the color has been established fix the slice in 10% formalin for ~20 minutes. The living tissue is colored and the infarcted tissue is a pale tan color[106107]. For the planimetry of the infarcts we used a simple program called Mobile Infarct Tool program a free shareware.[108] The most popular method for expressing infarct size is as a percentage of the region at risk. In that case the volume of the infarct for a heart is simply divided by the volume of the risk zone. Rats have negligible collateral flow and therefore, collateral flow need not be measured in these species.

3.2.8. Quantification of plasma GST enzyme

For measurement the alpha-GST concentration in serum we applied Rat alpha-GST ELISA kit (Abnova, alpha-GST (rat) Elisa kit, Cat. number: KA2134), following the manufacturers protocol. This method determines the free i.e. biological active alpha-GST concentration.

3.2.9. The measurement of oxidative stress parameters:

3.2.9.1. Analysis of malondialdehyde (MDA)

MDA is used for the quantification of lipid peroxidation in cell membranes. It was determined in anticoagulated whole blood or plasma, by photometric method[109].

3.2.9.2. Measurement of reduced glutathione (GSH) and plasma thiol (SH) groups

GSH and plasma SH levels were determined from anticoagulated whole blood (ethylene diamine tetraacetic acid (EDTA)) by Ellman's reagent according to the method of Sedlak and Lindsay[110].

3.2.9.3. Measurement of Superoxide dismutase (SOD) activity in washed red blood cell (RBC)

The main point of this measurement was that adrenaline is able to spontaneously transform to adrenochrome (a detectable colorful complex). This transformation can be blocked by SOD, and SOD containing cells or tissues. The difference in the rate of rise of control and sample curves obtained at 415 nm, are proportional to SOD activity[111].

3.2.9.4. Serum myeloperoxidase assay

Anticoagulated blood was centrifuged with 2000g, and 200 µl plasma was mixed with 1 ml working solution (0,1 M sodium-citrate 10,9 ml, 0,05% Triton-X 100 5 µl, 1mM H2O2 1 ml, 0,1% o-dianisidine 100 µl). The mixture was incubated at 37 ºC for 5 minutes, then 1 ml 35%

perchloric acid was added. Photometry were done at 560 nm. Plasma myeloperoxidase was expressed as nM/l. Hematologic measurement: Red blood cell count, white blood cell count, platelet numbers, haemoglobin concentration, haematocrit level were measured by Minitron automatic analysator (Diatron Ltd, Budapest, Hungary).

3.2.10. Serum TNF-alpha quantification

For measurement the TNF-alpha concentration in serum we applied Rat TNF-alpha ELISA kit (R&D Systems, Inc., Minneapolis, USA), following the manufacturers protocol. This method determines the free i.e. biological active TNF-alpha concentration.

3.2.11. Serum IL-1 quantification

For measuring the Interleukin 1 (IL-1) concentration in serum we used Rat IL-1 ELISA kit (R&D Systems, Inc., Minneapolis, USA), following the manufacturers protocol. This method determines the free i.e. biological active IL-1 concentration.

3.2.12. The western-blot analysis of pro/antiapoptotic (p38, ERK, Bad, GSK3ß) signaling pathways

We examine the effects of myocardial infarction and EA treatment and PC on the signaling pathways in the heart muscle. Fifty milligrams of frozen heart 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 with bovine serum albumin as a standard 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 electrophoretically 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-p38 MAPK (Thr180/Tyr182 , 1:1000 dilution), phospho-ERK MAPK (1:1000 dilution) , phospho-Bad (1:1000 dilution) and GSK3ß (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 peroxidaseconjugated secondary antibody (1:3000 dilution; Bio- Rad, Budapest, Hungary) for 1 h at room temperature. Membranes were washed six times for 5 min in TBST and the sites of antibody-antigen reaction were visualized, and the detection of signal was determined by using enhanced chemiluminescence (ECL Plus, Amersham, Piscataway, NJ) before exposure to photographic film. The developed films were scanned, and the band densities were quantified by means of Scion Image Beta 4.02 program. All experiments were repeated four times. Data for ERK and phospho- ERK represent the sum of the 42- and 44-kDa bands for each sample. For all experiments, control minigels were run before Western blot analysis and stained with Coomassie brilliant blue (Bio-Rad; Hercules, CA), and several representative bands were quantified by densitometry to ensure equality of loading. Equal protein loading in each lane was confirmed by probing for -actin. Adequate transfer of proteins from the gel to the membrane was confirmed by Coomassie blue staining of the gel and Ponceau red staining (Sigma-Aldrich Ltd, Budapest, Hungary) of the membrane.

3.2.13. Statistical analysis

All values are expressed as means ± standard error of the mean (S.E.M). Statistical comparisons include two samples Student t-test and one-way analysis of variance (ANOVA). $p<0.05$ was considered significant. Data presentation of Western blots are representative of series with similar results. Densitometric values are the mean±S.D. for the indicated number of independent experiments. Significance of differences was determined using ANOVA testing applying Bonferroni corrections for multiple samples. P values <0.05 were considered to be significant. Significant differences considered as relevant to major findings are marked in the graphs and their corresponding P values are indicated in the figure legend.

3.3. RESULTS

3.3.1. Haemodinamics

Changes in heart rate, systolic and diastolic blood pressures are shown in Table 1. The control, sham-operated, EA treated, AMI suffered, ischaemic PC and the combinated groups (AMI+EA, AMI+EA+PC**)** mean values are shown. Values were checked for significant variation during the time course of the experiment within any given group, as well as with the control group. Changes in heart rate did not show any significant fluctuations, even though most frequent rhythm disturbances were noted during early reperfusion. Similarly systolic and diastolic blood pressures only showed slight variations, however none of the changes were statistically significant. Although EA has been associated with a slight fall in mean arterial pressure in the treated groups, this was not relevance in our study. Haemodynamic parameters were comparable across the groups and it is unlikely that changes in infarct size could be attributed to haemodynamic variations (**Table 1.**)

	Heart rate (beat/min)	**Systolic pressure (mmHg)**	**Diastolic pressure (mmHg)**
Control	289±7	104±4	83±3
Sham	396±10	115±3	90±2
EA	295±3	98±4	76±3
AMI	415±9	128±3	96±2
PC	385±5	113±2	90±3
AMI+EA	394±5	117±2	92±2
AMI+EA+PC	396±6	114±3	91±2

Table 1. Haemodynamic variations in the different groups. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.2. Measuring infarct size by the tetrazolium method

The infarction lesion size varied from 8,7% to 31,7% of total cardiac muscle (cross section). Infarct size as a percentage of the total cardiac muscle is demonstrated in **Figure 4**. The infarct size was significantly ($p<0.01$) different in every groups (PC,AMI+EA,AMI+EA+PC) comparing to the AMI group. The AMI+EA group had a mean infarct size of 28,3 ± 3% of the total muscle. PC limited the infarction in groups PC and AMI+EA+PC compared to the groups AMI and AMI+EA(slightly but not significantly). The infarction size was significantly higher in the presence of EA in the AMI+EA+PC group compared to the PC (10.3 ± 1 % vs. 26.9 ± 3 %) ($p<0.01$).

The differencies between the stained and non-stained areas are illustrated in **Picture 1**.

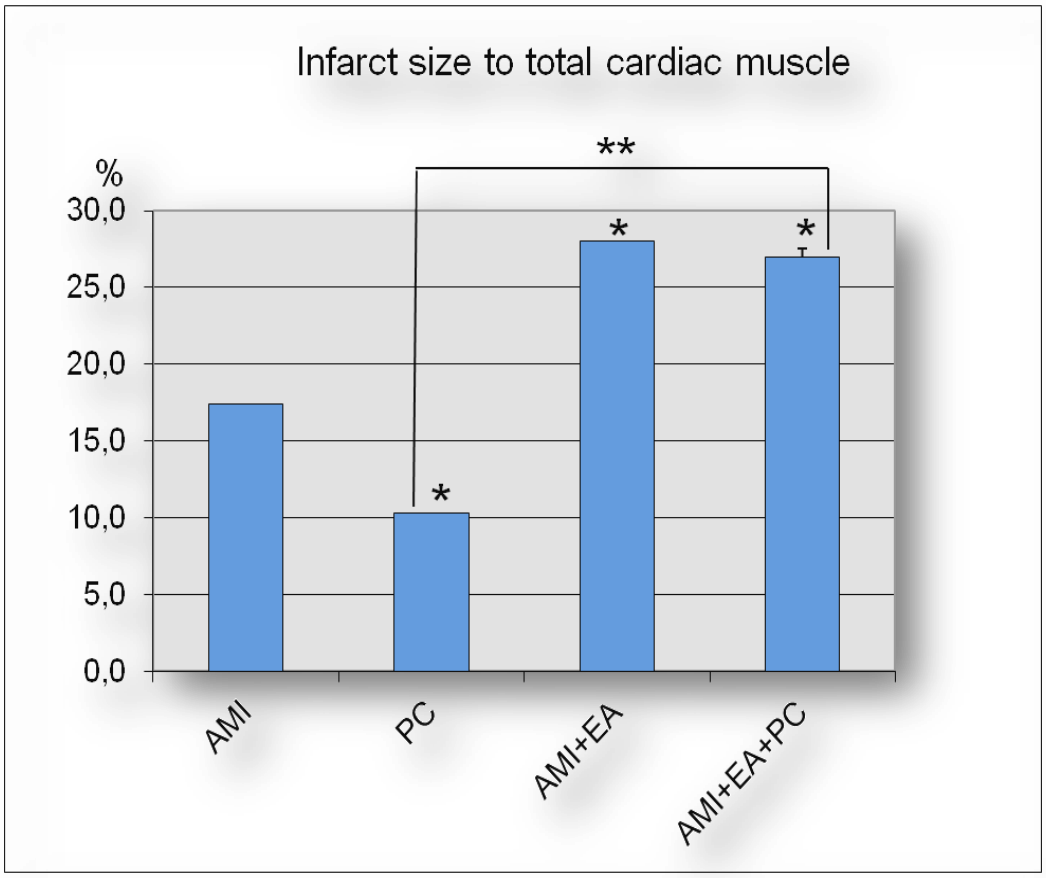
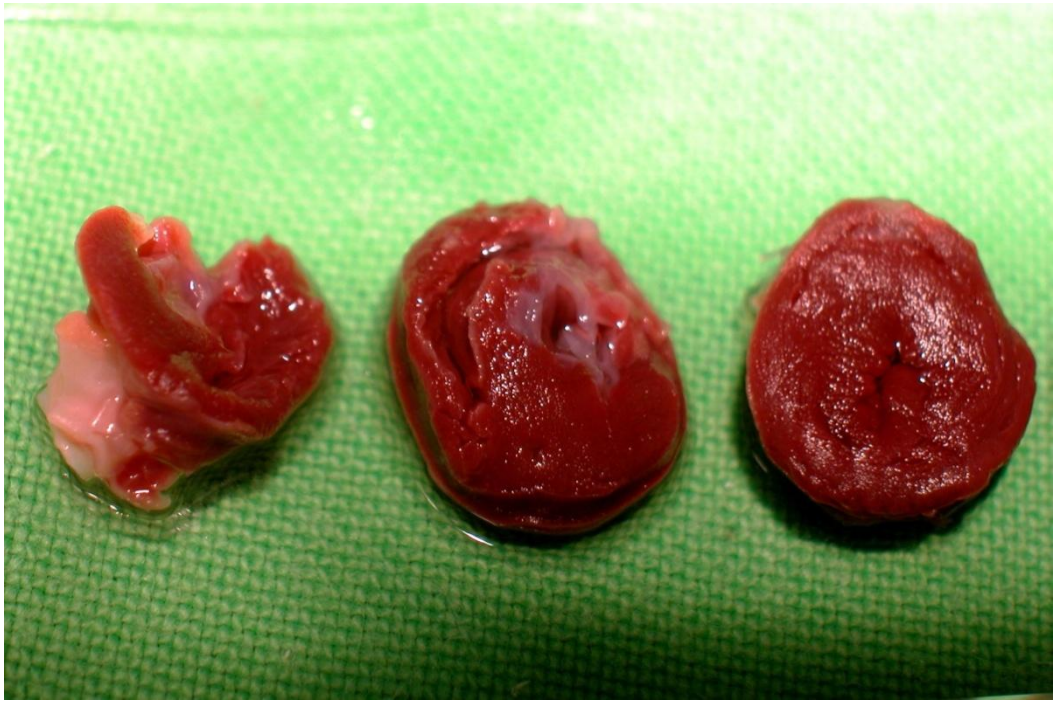


Figure 4. Size of acute myocardial infarction between the groups. *p<0,01 compared with the AMI group. **p<0,01 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.



Picture 1. Measuring infarct size by the tetrazolium method. The ischemic area remained sallow while the non-infacted tissues stained in red.

3.3.3. Quantification of plasma GST enzyme

The GST concentration in the plasma was significantly lower in two groups comparing to the control group (*p<0.05 control vs. AMI+EA, 67,5±4,8ng/ml vs 46,0±8,5ng/ml **p<0.01 control vs. EA 67,5±4,8ng/ml vs 36,1±2). In the PC group there was a significantly (*p<0.05) raising of plasma GST compared to the AMI group. Our data showed significant (*p<0.05) differences between PC and AMI+EA+PC groups. GST level was significantly lower in the presence of EA (69,3 ± 9,8 ng/ml vs. 52,6 ± 6,6 ng/ml).(**Figure 5**.)

The GST concentration was more markedly lower in AMI (p<0.01), AMI+EA (p<0.01) and AMI+EA+PC (p<0.05) groups compared to the sham group.

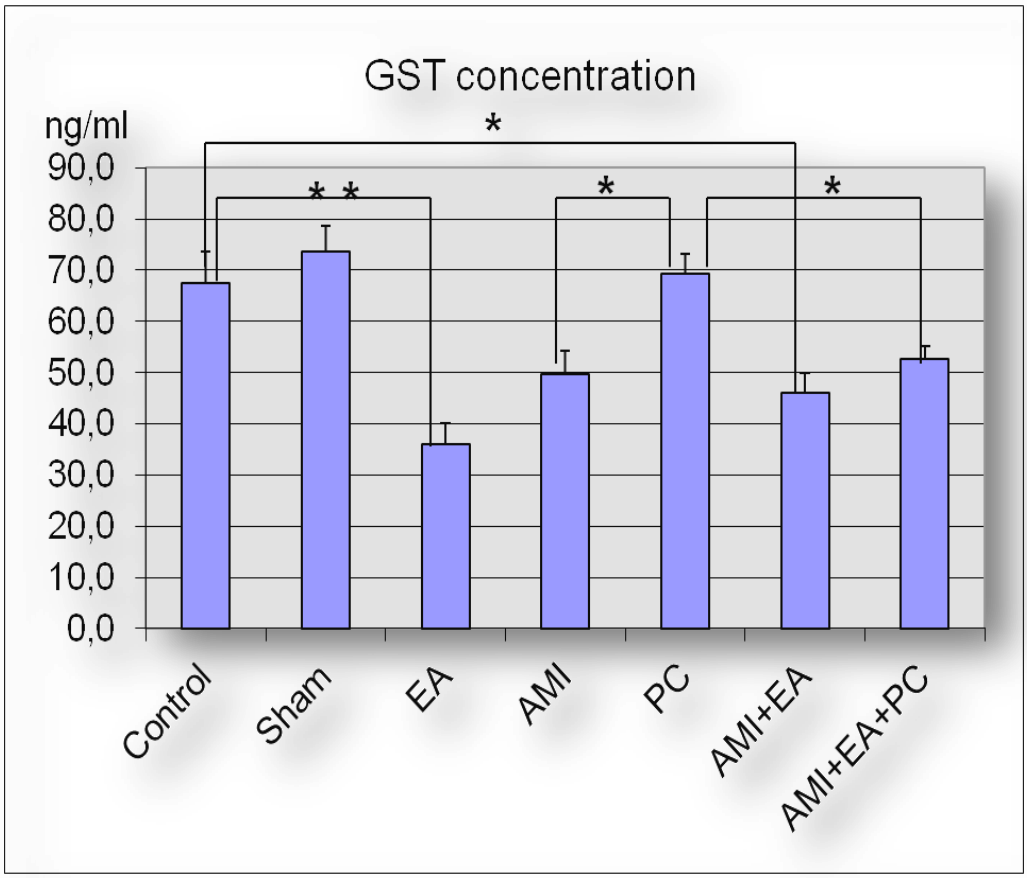


Figure 5. Serum GST concentration in the different groups. *p<0,05 compared the linked groups to each other. **p<0,01 compared with the control group. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.4. Malondialdehyde and plasma malondialdehyde levels

In our experiment we measured the values of MDA level indicating membrane damage and lipidperoxidation in an in vivo animal model. The MDA concentration was significantly ($p<0.05$) higher in five groups (EA, AMI, PC, AMI+EA, AMI+EA+PC) comparing to the control group. Our data showed significant ($p<0.05$) differences between PC and AMI+EA+PC groups. MDA level was significantly higher in the AMI+EA+PC group (72.7 ± 2 nmol/ml vs. 78.3 ± 1 nmol/ml).

We found significantly ($p<0.05$) lower level of MDA in AMI+EA+PC group comparing to the AMI+EA group (78.3 ± 1 nmol/ml vs. 82.1 ± 1.5 nmol/ml). The membrane-damaging

effect of AMI was elevated but could not reach the level of significancy in the presence of EA. (**Figure 6.**)

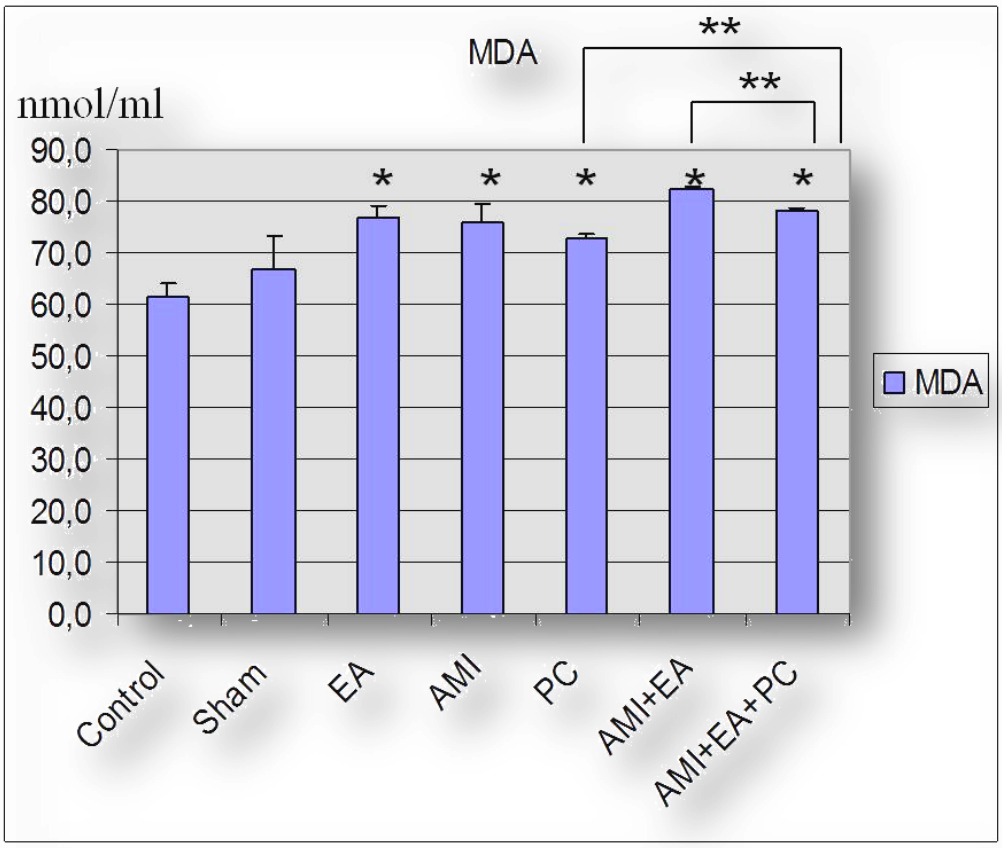


Figure 6. Changes in malondialdehyde concentration between the groups. *p<0,05 compared with the control group. **p<0,05 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

The MDA concentration in the plasma was significantly higher in five groups (*p<0.05 EA, **p<0.01 AMI, PC, AMI+EA, AMI+EA+PC) comparing to the control group. Compared the values to the sham group we found the same significances. In the PC group there was a strongly significant (p<0.01) decrease of plasma MDA compared to the AMI group. The membrane-damaging effect of AMI was significantly elevated in the presence of EA (0.61±0.02 vs. 0.28 ±0.03). Our data showed strongly significant (p<0.01) differences between PC and AMI+EA+PC groups. MDA level was significantly higher in the presence of EA (0.34 ± 0.03 nmol/ml vs. 0.65 ± 0.03 nmol/ml). We found significantly (p<0.05) lower

level of MDA in AMI+EA+PC group comparing to the AMI+EA group (0.65 ± 0.03 nmol/ml vs. 0.96 ± 0.13 nmol/ml). (**Figure 7.**)

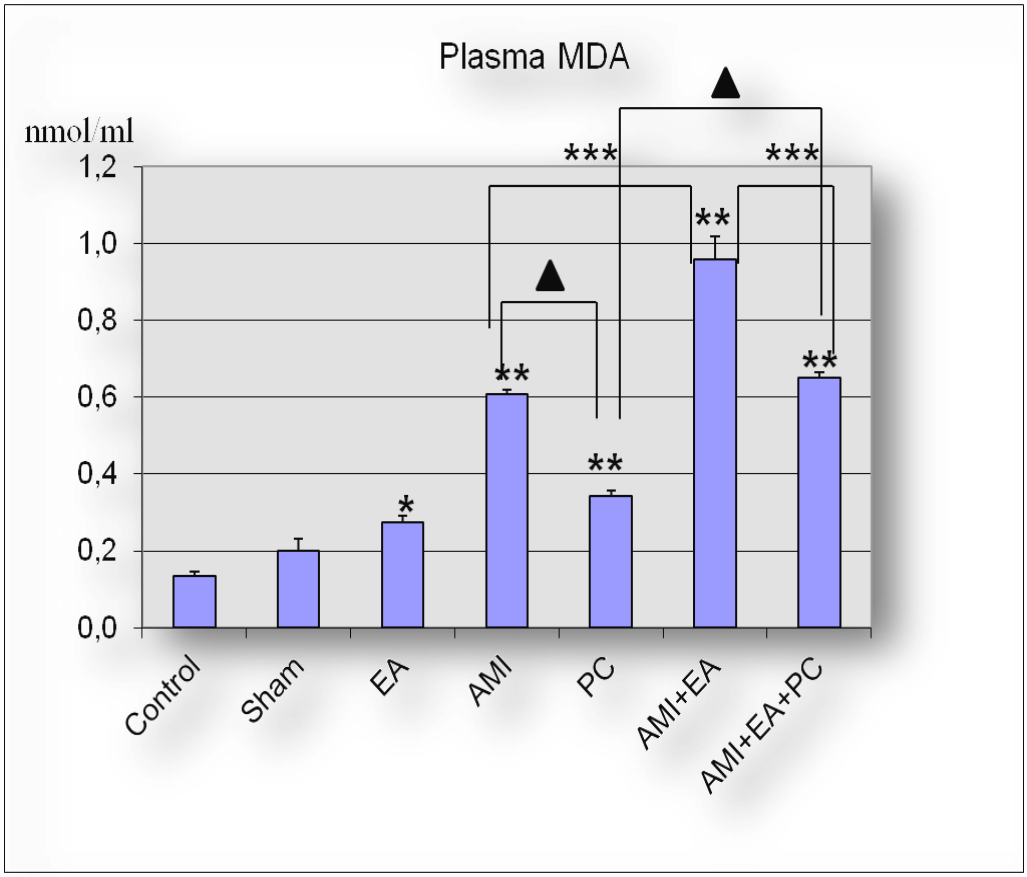


Figure 7. Changes in plasma malondialdehyde concentration between the groups. *p<0,05 , **p<0,01 compared with the control group. ***p<0,05 compared the linked groups to each other. ▲p<0,01 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.5. Reduced glutathione levels (GSH)

The values of reduced GSH levels were significantly ($p<0.05$) lower in two groups (PC, AMI+EA) comparing to the control group. In postconditioned EA treated (AMI+EA+PC) group the values were significantly higher than in non-conditioned EA (AMI+EA) group (802.4 ± 8.5 nmol/ml vs. 768.0 ± 20.0 nmol/ml), however this protecting factor of postconditioning between the similar groups in the presence of EA was not significant. The GSH level in the EA treated AMI group was definitely decreased compared to the AMI group without significance. (**Figure 8.**)

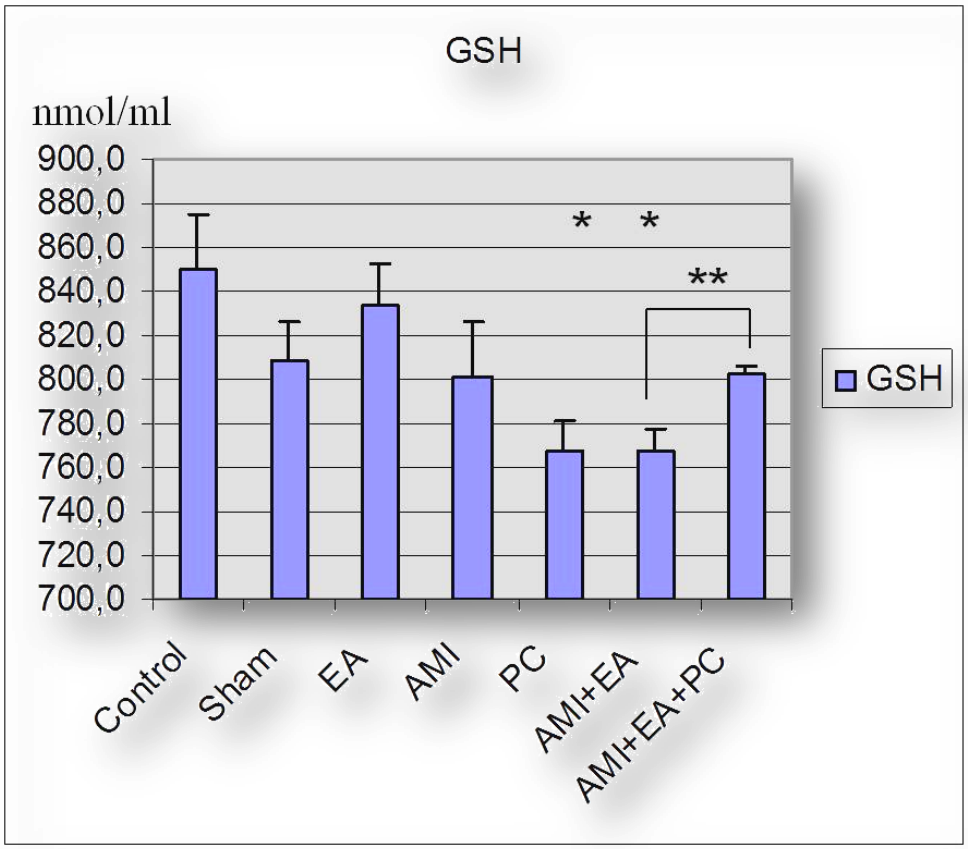


Figure 8. Plasma levels of reduced glutathione (GSH) between the groups. *p<0,05 compared with the control group. **p<0,05 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.6. Plasma thiol-groups (-SH)

We detected in the AMI+EA group significantly lower levels of –SH comparing to control group. We found significantly ($p<0.05$) lower level of -SH in AMI+EA group comparing to the postconditioned AMI+EA group. We found in the AMI+EA and AMI+EA+PC groups lower values than in similar groups without administration of EA (AMI, PC). There was not significant difference in –SH level between other groups. (**Figure 9.**)

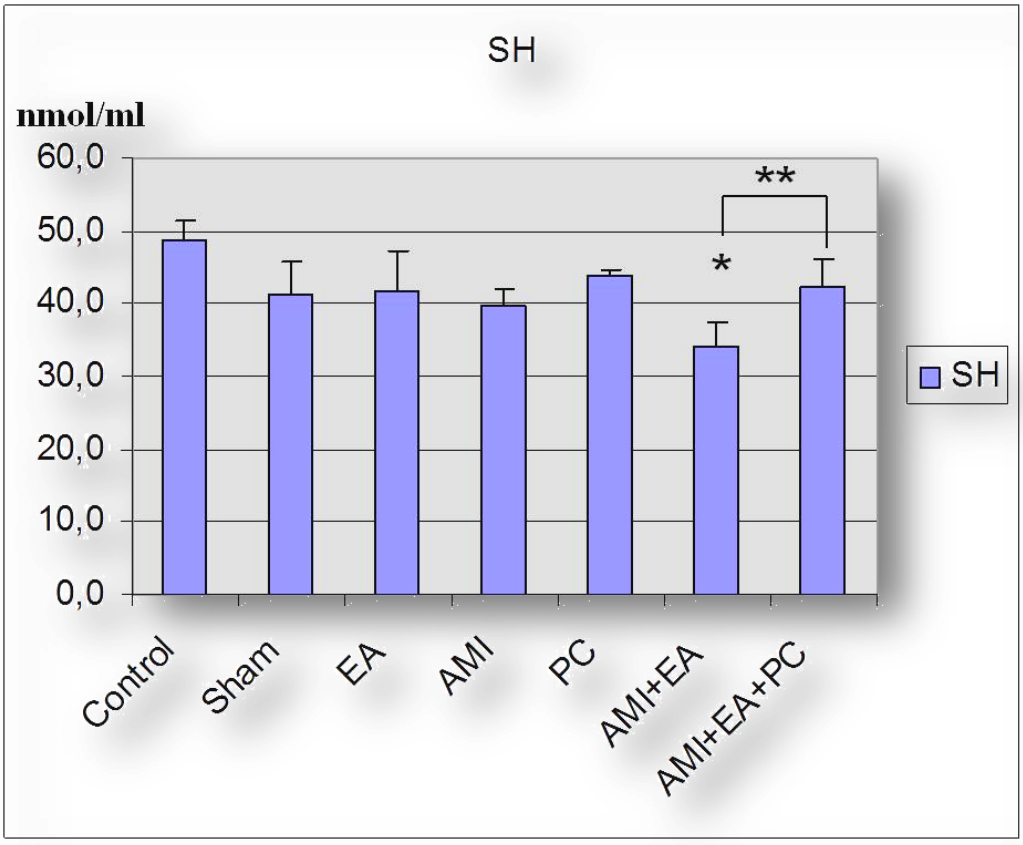


Figure 9. Changes in thiol (-SH) group plasma levels between the examined groups. *p<0,05 compared with the control group. **p<0,05 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.7. Enzyme activity of superoxide dismutase (SOD)

The SOD concentration was significantly ($p<0.05$) higher in two groups (Sham, AMI+EA+PC) and besides significantly lower in two groups (EA, AMI+EA) comparing to the control group. The concentration of SOD was more markedly lower in AMI+EA ($p<0.01$) group compared to the sham group. Our data showed significant ($p<0.05$) differences between AMI and AMI+EA groups. SOD level was significantly higher in the absence of EA (1137.7 ± 323.7 U/L vs. 682.6 ± 52.8 U/L). We found a strongly significant correlation between the AMI+EA and AMI+EA+PC groups. ($p<0.01$). The amount of SOD were sharply increased when the animals were postconditionated. (682.6 ± 52.8 U/L vs. 1331.8 ±181.0 U/L). (**Figure 10.**)

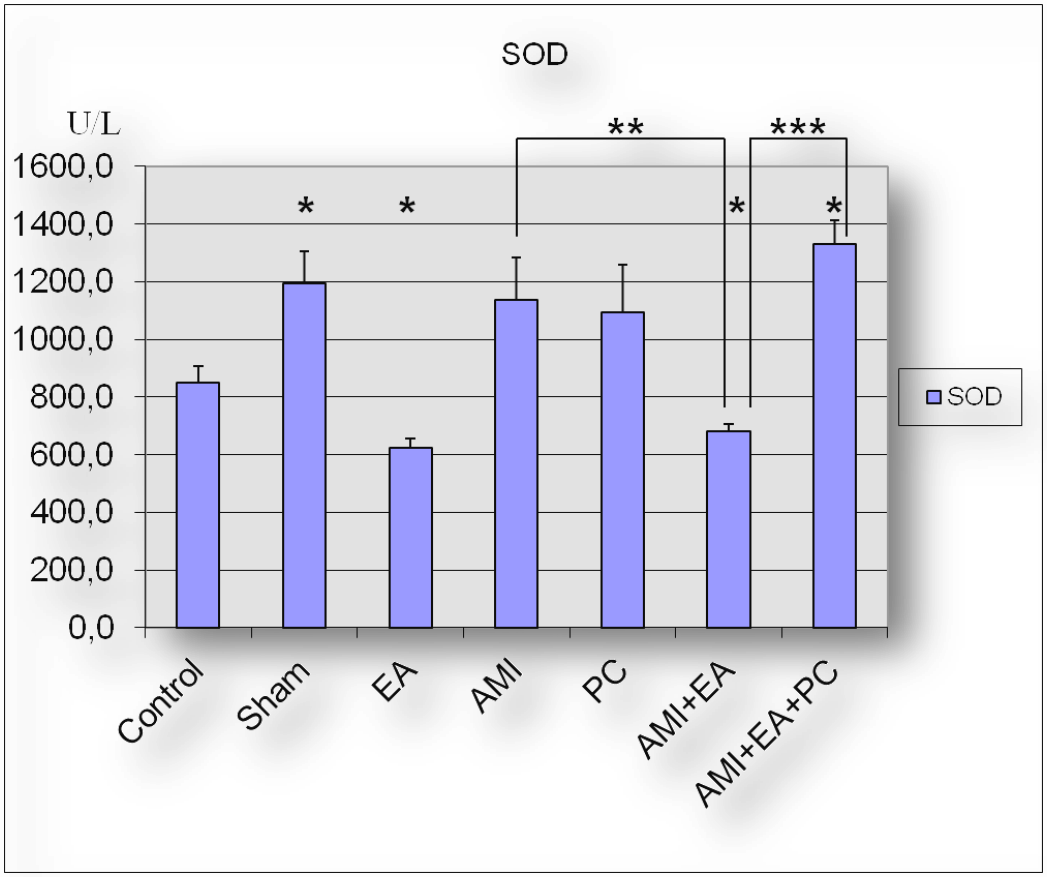


Figure 10. Changes in blood superoxide dismutase activity during the examined groups. *p<0,05 compared with the control group. **p<0,05 compared the linked groups to each other. ***<0,01 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.8. Serum myeloperoxidase results

To characterize the neutrophyl activation we measured the plasma myeloperoxidase (MPO) level and the induced ROS production of leukocytes. MPO level increased significantly in every group compared to the control group (*p<0,05 Sham, EA, AMI, PC, AMI+EA+PC, **p<0,01 AMI+EA group). Compared the values to the sham group we found the same significances. In the postconditioned group there was significantly lower level of MPO, in the EA treated AMI group a strongly significantly higher level of MPO compared to the AMI group (AMI:0.3±0.1 μM/ml vs PC:0.13 ±0.02 μM/ml. AMI+EA:0.6±0.1 μM/ml). To compare the values of PC and EA-treated postconditioned (AMI+EA+PC) groups we found a significant difference (0.13 ± 0.02 μM/ml vs 0.29 ± 0.04 μM/ml p<0.05). When we were postconditioning the EA treated AMI group, it was seen a strongly significantly lower MPO level (AMI+EA: 0.6±0.1 μM/ml vs. AMI+EA+PC: 0.29 ± 0.04 μM/ml) (**Figure 11**)

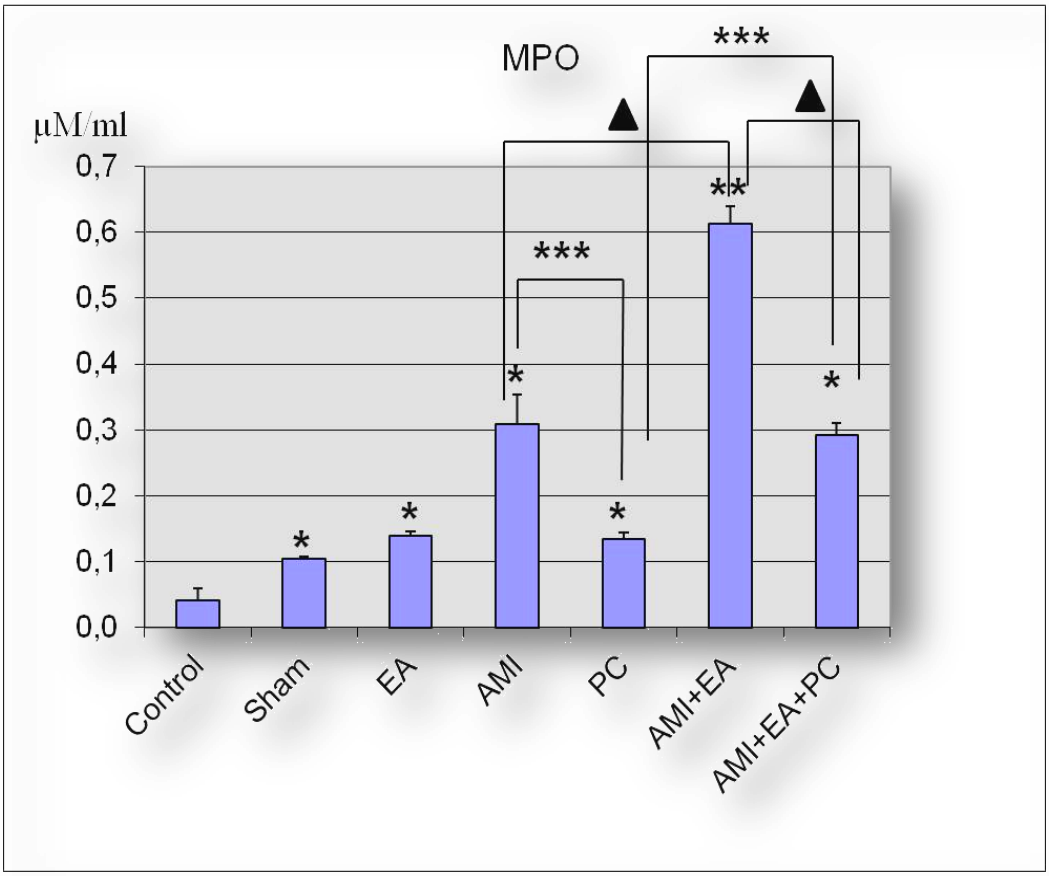


Figure 11: Serum myeloperoxidase (MPO) concentration in the different groups. *p<0,05 compared with the control group. **p<0,01 compared with the control group, ***p<0,05 compared the linked groups to each other. ▲p<0,01 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.9. Serum TNF-alpha levels

In the study we measured the TNF-alpha levels in the groups. The values were significantly higher (p<0.05) in five groups (EA, AMI, PC, AMI+EA, AMI+EA+PC) than in the control group (26,1 ± 3.7 pg/ml, 27.9 ± 2.9 pg/ml, 24 ± 1.7 pg/ml, 25,6±2,4 pg/ml, 25,3±1,5 pg/ml vs. 17.9 ± 0.7 pg/ml). In the sham operated group we have not found significant elevation in the level of TNF-alpha comparing to the control group (24.6 ± 6 pg/ml vs. 17.9 ± 0.7 pg/ml). The EA treatment itself caused damage moreover the protecting effect of PC was not marked in the presence of EA. (**Figure 12.**)

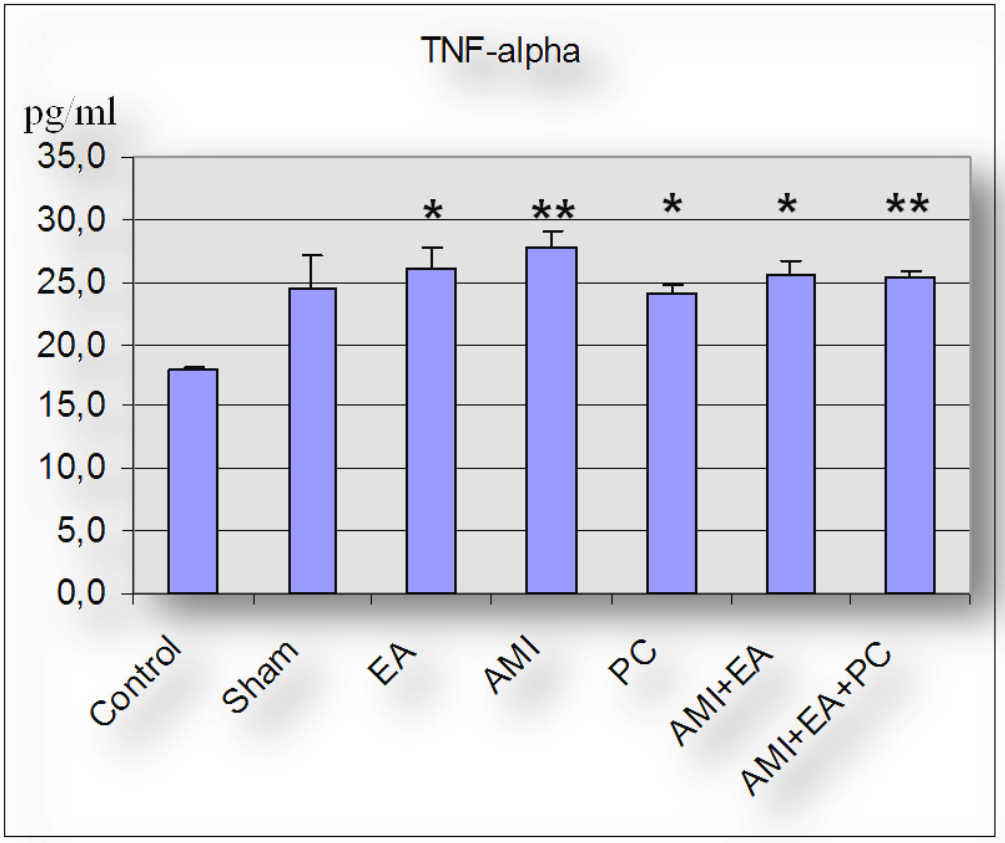


Figure 12. TNF-alpha concentrations show the grade of inflammatory response in the groups.*p<0,05 compared with the control group. **p<0,01 compared with the control group. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.10. Serum interleukin-1 (IL-1)

We investigated the serum IL-1 levels in our groups. The values were significantly higher in three groups (EA, AMI+EA, AMI+EA+PC) than in the control group. These groups are all EA treated groups. When this EA treatment was combined with AMI, the significance was eve more apparent compared to the control. Postconditioning did not temper significantly that elevation of IL-1 level if the animals were co-treated with EA administration. Compared the values to the sham group we found the same significances. (**Figure 13.**)

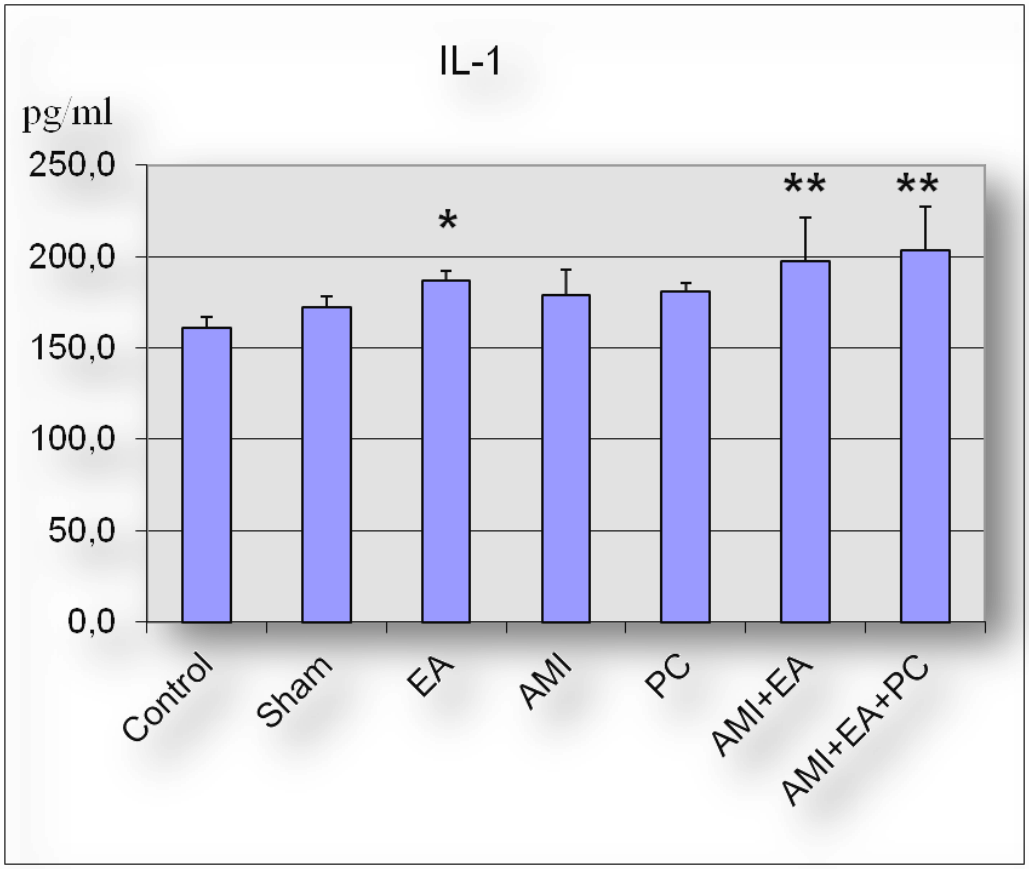
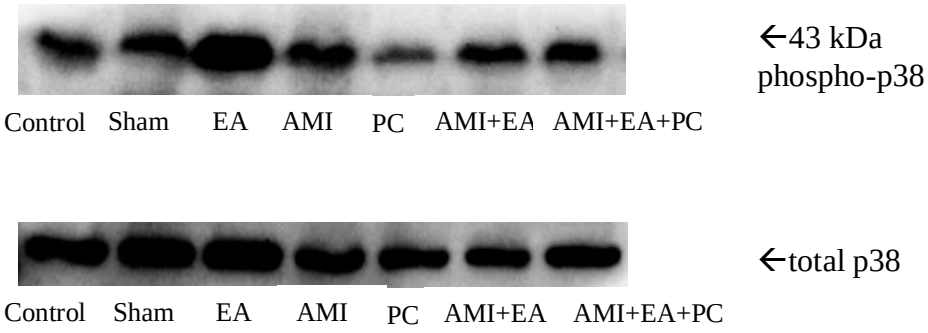


Figure 13. IL-1 plasma-concentrations show the grade of inflammatory response in the groups. *p<0,05 compared with the control group. **p<0,01 compared with the control group. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning.

3.3.11. The western-blot analysis of pro/antiapoptotic signaling pathways

Phosphorilation of **p38 MAPK** is demonstrated in **Figure 14-15**. The activation was significantly (p<0.001) higher in four groups (AMI, EA, AMI+EA, AMI+EA+PC) comparing to the control group. Compared the values to the sham group we found the same significances. Postconditioning limited the phosphorilation of p38 in groups PC and AMI+EA+PC compared to the groups AMI and AMI+EA (p<0.001). The presence of EA increased the level of activated p38 in the AMI+EA and AMI+EA+PC groups compared to the AMI and PC groups. (p#<0,01). Significant decrease in p38 MAPK activation was found in the EA and PC treated group compared to the AMI group.

The activated phospho-p38 MAPK is illustrated in **Figure 14.**



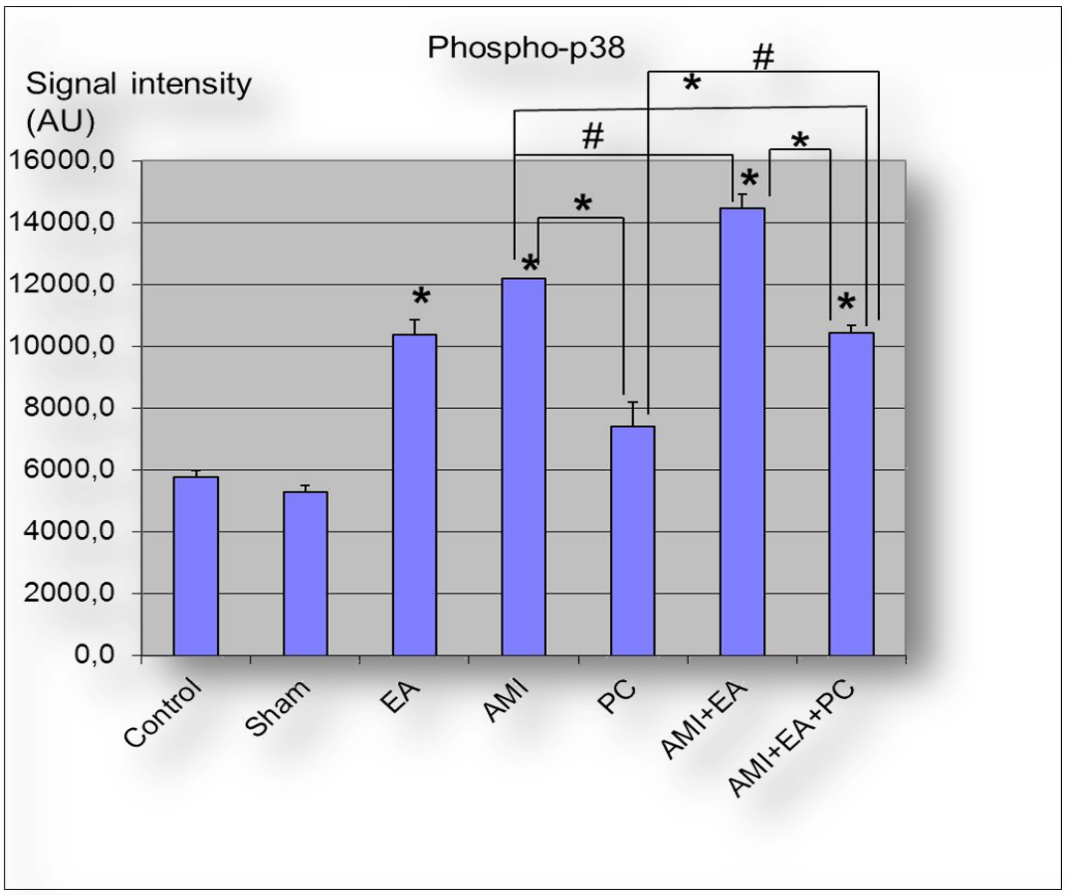
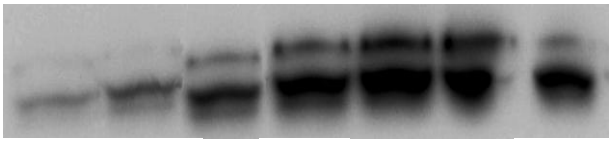


Figure 15. Phosphorylation of p38 mitogen-activated protein kinase p*<0,001 compared with the control group or compared the linked groups to each other, p#<0,01 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning, AU Arbitrary Units. The phosphorylation levels of p38 were normalized to the levels of total p38.

ERK phosphorylation strongly increased in every group (**p<0,001) compared with the control group, except the sham-operated group where weak increase can be observed (*p<0,05). In the PC (#p<0.01) and AMI+EA (▲p<0,05) group there was a significantly raising of activation of ERK compared to the AMI group. Our data showed significant differences AMI, PC (€p<0,001) and AMI+EA (#p<0,01) groups compared to the AMI+EA+PC group. The active phosphorilated form of ERK was significantly lower when AMI suffered animals were cotreated with EA and PC. (**Figure 16-17**.)

Figure 16. Phosphorylation of ERK:



←p ERK 44/42 kDa

Control Sham EA AMI PC AMI+EA AMI+EA+PC

←total ERK

Control Sham EA AMI PC AMI+EA AMI+EA+PC

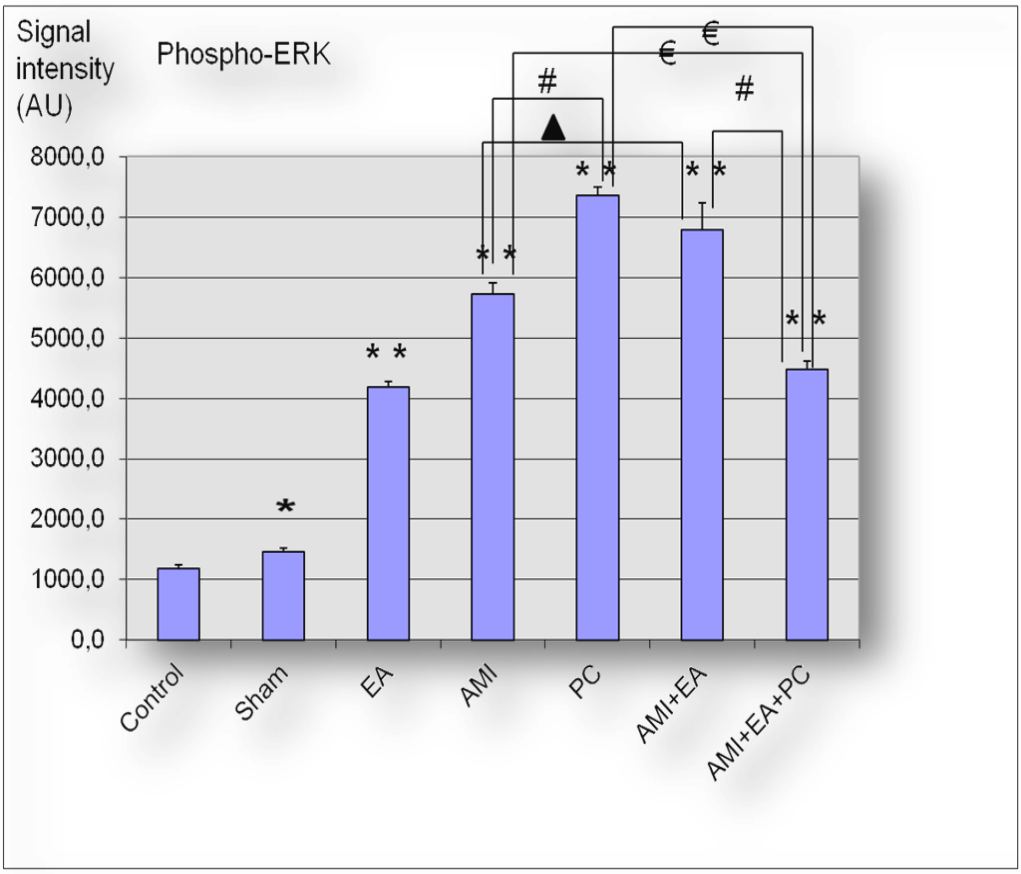
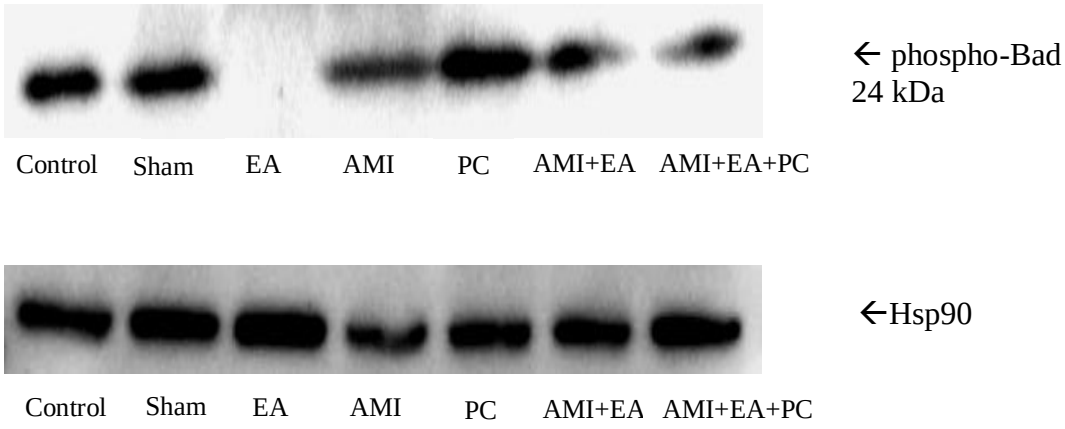


Figure 17. Phosphorylation of ERK. *p<0,05 compared with the control group, **p<0,001 compared with the control group, ▲p<0,05 compared the linked groups to each other, #p<0,01 compared the linked groups to each other, €p<0,001 compared the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning, AU Arbitrary Units. The phosphorylation levels of ERK were normalized to the levels of total ERK.

In this case we measured the values of **phosphorilated Bad** level. Proapoptotic activity of Bad is inhibited through its phosphorylation. The p-Bad concentration was significantly different in five groups (AMI (*p<0.05), PC, AMI+EA(**p<0.01), EA, AMI+EA+PC(#p<0.001)) comparing to the control group. Compared the values to the sham group we found the same significances. Our data showed significant differences between AMI and AMI+EA (*p<0.05) and between AMI and AMI+EA+PC groups (**p<0.01). In the presence of EA there was a decreased phosphorylation observed which has worsened the cell survival. On the contrary the postconditioning increased the p-Bad compared to the AMI

group (**p<0.01). We found significantly lower level of p-Bad in AMI+EA+PC group comparing to the PC group (#p<0.001). The protective effect of PC seems to be weaker in the present of EA. (**Figure 18-19.**)

Figure 18. Phosphorylation of Bad



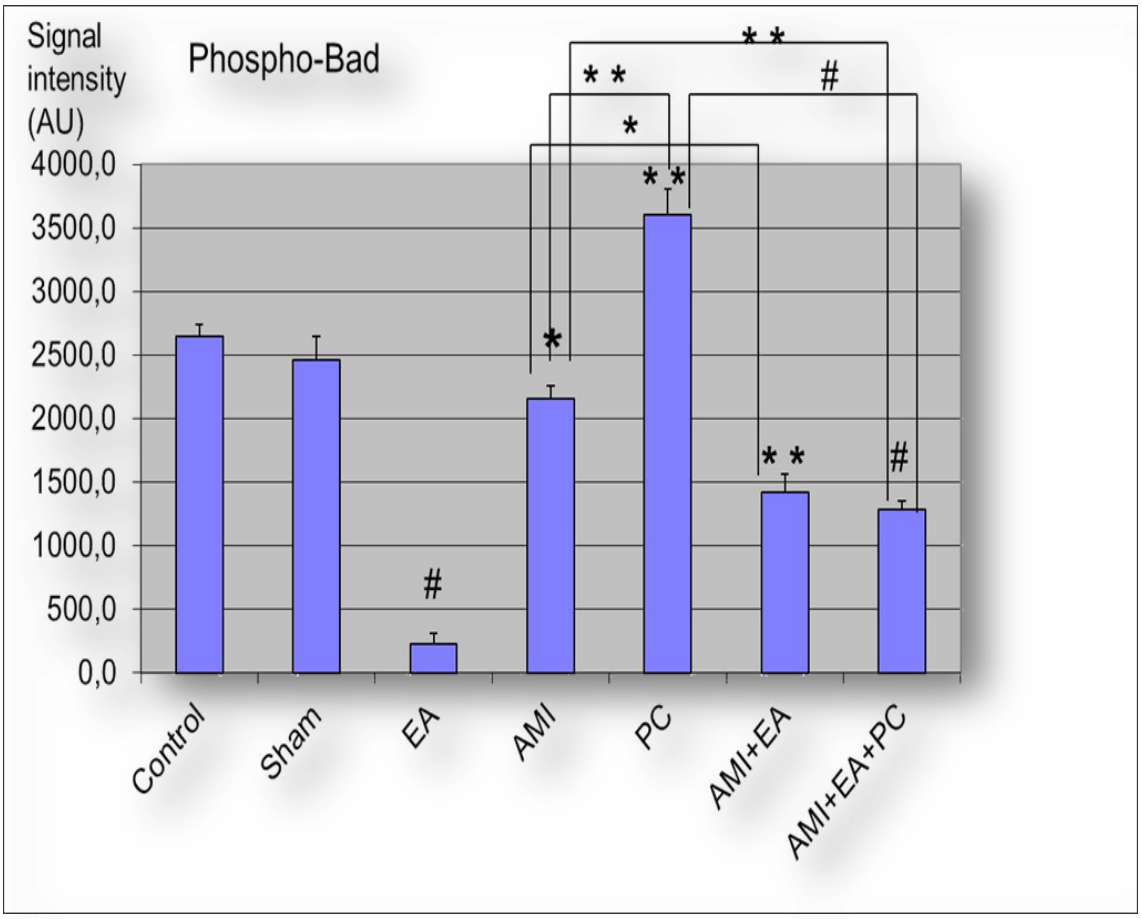


Figure 19. Phosphorylation of Bad. *p<0,05 compared with the control group or the linked groups to each other, **p<0,01 compared with the control group or the linked groups to each other, #p<0,001 compared with the control group or the linked groups to each other. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning, AU Arbitrary Units.

The **GSK-3ß** concentration was significantly higher in five groups (*p<0.05 EA, AMI, PC, AMI+EA+PC **p<0.01 AMI+EA) comparing to the control group. Compared the values to the sham group we found the same significances. Our data showed significantly (*p<0.05) lower level of GSK-3ß in AMI+EA+PC group comparing to the AMI+EA group. (**Figure 20-21.**)

Figure 20. Total amount of GSK3ß

←47 kDa
total GSK3ß

Control Sham EA AMI PC AMI+EA AMI+EA+PC

←Hsp90

Control Sham EA AMI PC AMI+EA AMI+EA+PC

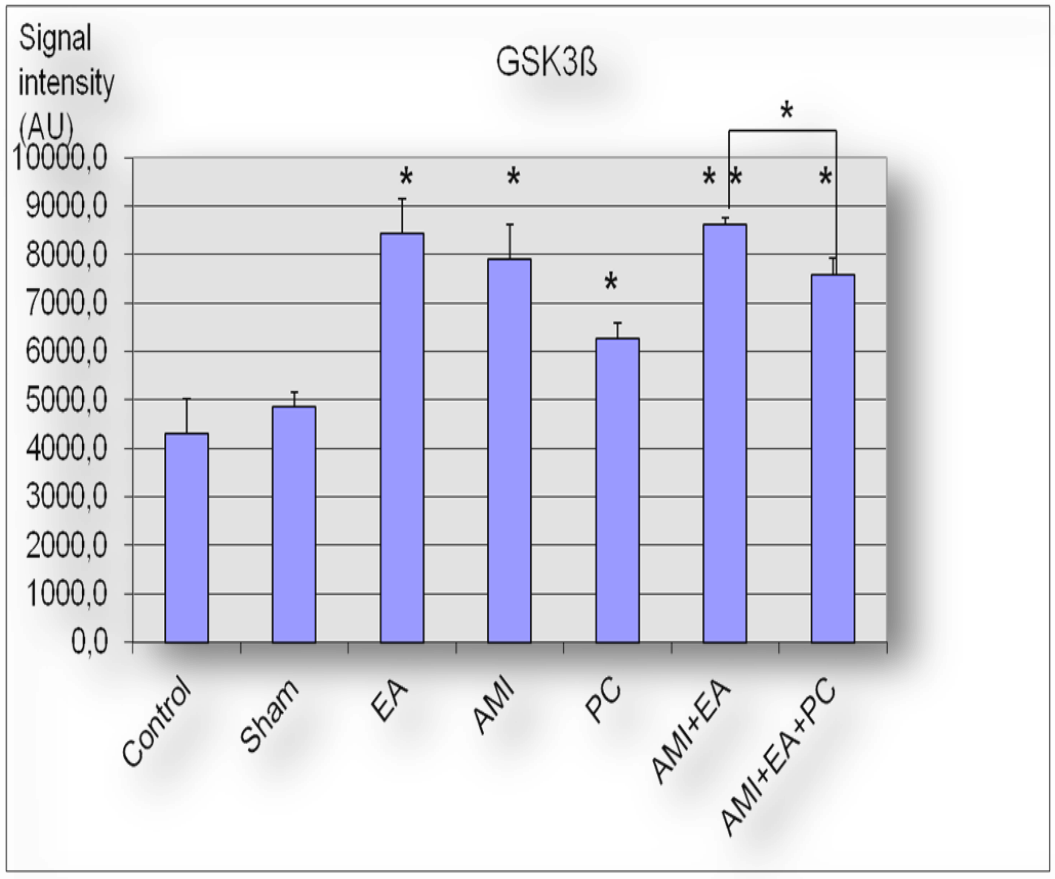


Figure 21. Total amount of GSK3ß. *p<0,05 compared with the control group or the linked groups to each other, **p<0,01 compared with the control group. EA Ethacrynic acid; AMI acute myocardial infaction; PC postconditioning, AU Arbitrary Units.

3.4. DISCUSSION

Measuring the plasma GST levels we were able to demonstrate that EA is working as an effective pharmacological inhibitor of GST and could markedly magnify oxidative stress-induced apoptosis in our in vivo model. It was reported double stress when animals simultaneously underwent a myocardial infarction. Our results were more confirmed by the measurement of total infarct size. The infarction size was significantly higher in the presence of EA while PC limited the extent of infarcted area. Using GST inhibition during AMI (EA+AMI) the protective effect of postconditioning is lost.

Hypoxia will lead to decreasing level of intracellular ATP and continuous increase of hypoxanthine. In the very early moments of reperfusion the oxygen is not present in the cell and the xanthine oxidase catalised hypoxanthine-xanthine conversion will produce a mass of superoxide radicals. Through lipid peroxidation the reactive oxygen intermediates (ROI) and other superoxide radicals will damage the membrane lipids, proteins and DNA. The endogenous antioxidant system tries to protect the cells and macromolecules against these injuries.[112] We have found that myocardial infarction caused I/R cause increased oxidative stress parameters which was further increased by administration of EA. The positive effect of ischaemic PC have been detected through the investigation of oxidative parameters and this positive effect has seen in the presence of EA (AMI+EA+PC). The increased level of ROS and a more disadvantageous GSH state may overact the intensity of insult and may explain the increased amount of apoptotic cells in GST-inhibited groups during myocardial I/R.

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-1), produced by both macrophages and neutrophils, is elevated in the ischaemic heart and may have a pathophysiological role following I/R injury.[113] TNF-α induces three pathways: apoptotic cell death; activation of MAPK pathway; and NFKB-pathway.[114] Our results indicate that GST inhibition itself is able to increase the levels of the proinflammatory cytokines and the protective effect of PC can not prevail.

GSTs play an important role as antioxidant enzyme modulating MAPK and RISK pathways moreover GST inhibition was associated with increased activation of MAP and RISK kinases under stress conditions.[115]

The MAPK proapoptotic signalling pathway through p38 is activated by oxidative stress and is associated with cellular damage, mediation stress response and cytokine production. We have found that oxidative I/R cause noticeable induction of p38 activity in ischemic myocardium, which is further increased by EA administration. Moreover we have found that pharmacological inhibition of GST augments p38 activity itself. We were able to strengthen the protective effect (the decreased phosphorilation of p38) of PC alone. The PC was able to decrease the phosphorylation of p38 in the group where animals were exposed to double stress as well (AMI+EA+PC vs. AMI+EA). The GST inhibition decreased the protective effect of PC (PC vs. AMI+EA+PC).

According to our results, ERK is activated in every group including on GST inhibition in the presence of EA administration. The level of phosphorylated ERK of GST-inhibited cells undergo AMI (AMI+EA) transcended the ERK phosphorylation level of cells that have undergone AMI alone. In our experiment the level of antiapoptotic phospho-ERK was significantly higher following PC – consistent with previous studies – but this change was not shown in the presence of GST inhibitor EA. Although the activation of ERK increased due to GST inhibition (AMI+EA) or PC the elevation of this antiapoptotic MAPK was lost in case of double stress (AMI+EA+PC), probably cause of the elevation of ERK induced by PC was stopped because of the presence of GST inhibitor EA.

Many studies have suggested that the MAPKs may be important regulators of apoptosis in response to myocardial I/R. In addition to this the present study showed that inhibition of GST by EA leads to increased phosphorilation of p38 proapoptotic and antiapoptotic ERK signaling proteins during myocardial I/R and what is more it was able to abolish the protective effect of PC. These findings represent the association between MAP kinases and GST during myocardial stress.

Phospho Bad activation decreased markedly upon administration of EA and in AMI groups or in the group suffered from double stress (AMI+EA) compared to the control. Ischaemic PC increased the Bad phosphorilation significantly in PC group while this change could not be detected in EA-treated simulated PC group (AMI+EA+PC) compared to the control. EA administration resulted in further decrease in Bad activation in animals exposed to myocardial

ischemia (AMI vs. AMI+EA) and even the PC was not able to repeair this damage (AMI vs. AMI+EA+PC). It was observed the same result between the AMI+EA vs. AMI+EA+PC groups. The PC was unable to correct the devastation, we were obtained similar high proapoptotic results in the two groups. Among the postconditioned groups the level of phosphorylated Bad was significantly lower when animals were treated with GST inhibitor EA (PC vs. AMI+EA+PC).

Myocardial infarction, GST inhibition (EA) led to significant increase in GSK-3β activation related to non-treated animals (control). EA administration during myocardial ischemia (AMI+EA) resulted in further elevation in activation of GSK-3β. A significant decrease in GSK-3β activation was detected in EA-treated ischaemic postconditioned group (AMI+EA+PC) compared to AMI+EA group. We were able to detect a markedly but not significantly increase of proapoptotic GSK-3β in the presence of GST inhibition (AMI+EA+PC) compared to the PC group. We can confirm our previous results that the EA is capable to reduce the favorable effect of PC.

Our study has been conducted to examine the biological role of GST in vivo myocardial infarction rat model. We found that pharmacological inhibition of GST by EA augments the apoptosis as a result of oxidative stress and myocardial ischaemic injury. The study showed that GST inhibition was associated with increased activation of MAP and RISK kinases under stress condition.

4. POLYMORPHISMS IN GLUTATHIONE S-TRANSFERASE ARE RISK FACTORS FOR PERIOPERATIVE ACUTE MYOCARDIAL INFARCTION AFTER CARDIAC SURGERY

4.1. INTRODUCTION

For revascularisation of multiple coronary artery disease CABG remains the primary procedure of choice /treatment option. Despite the improvement in surgical, anaesthetic technique and treatment modalities CABG is frequently complicated by PMI. PMI is associated with adverse outcome following cardiac surgery, reduced long-term survival and increased costs. The incidence of PMI varies considerably, from 3% to 30%, because of different diagnostic criteria and variable patient populations [116117118]. Multiple mechanisms play a role and interplay in pathomechanism of PMI, such as I/R during and following aortic cross clamping, furthermore cardiac manipulation, inadequate myocardial protection, and intraoperative defibrillation, and acute occlusion of bypass grafts [119120121]. On the other hand primary mechanisms in the pathogenesis of perioperative myonecrosis is associated with further factors, like complex acute inflammatory response, global ischemia, manipulation. Recent studies have highlighted the significance of inter-personal variability of PMI suggesting the role of genetic, hereditary component in pathomechanism[122 123].

Oxidative stress may occur as a consequence of multiple mechanisms during cardiac surgery resulting in myocardial injury[124125126127128]. Among antioxidant enzymes eliminating the potential harm of reactive oxidants, the GSTs may play crucial part.

The GSTP1 isoform is one of the most investigated isoenzyme which is widely expressed in different human epithelial tissues and GSTP1 is predominantly expressed in myocardial cells compared with other isoenzymes[129]. The gene of GSTP1 located on chromosome 11 has a wild type form named as GSTP1*A (Ile105Ile/Ala113Ala) and two other single nucleotide polymorphic forms named as GSTP1*B (Ile105Val/Ala113Ala) and GSTP1*C (Ile105Val/Ala113Val) [130]. These are designated as A, B and C alleles that are expressed in various homozygous and heterozygous combinations modifying the activity of enzyme.

At the level of enzyme one of the most active GST of liver is the products of a polymorphic autosomal loci GSTM1[131]. Over and above the enzyme can be easily detected in adult kidney, adrenal and stomach but is only weakly expressed in skeletal and cardiac muscle

and not at all in fetal liver, fibroblasts, erythrocytes, lymphocytes and platelets. GSTP1 is found in every tissue except adult liver[132].

The presence or absence of the GSTT1 gene was coincident with the conjugator (GSTT1+) and nonconjugator (GSTT1-) phenotypes, respectively. In humans, the GSTT1 enzyme is found in the erythrocyte and this may act as a detoxification pool.

Polymorphism altering or reducing these enzyme detoxification activities could increase a person's susceptibility to pathologies mediated by oxidative processes.

For both the GSTM1 and GSTT1 enzymes, the defect is due to a deletion of the entire genomic coding region, giving rise to the allele known as "null." The deletion in a homozygous state determines the polymorphism called "null genotype," with the consequent absence of total enzymatic activity.

In the present study it has been hypothesised that SNPs in GSTs are associated with the incidence of postoperative myocardial infarction in a cohort of patients undergoing cardiac surgery with cardiopulmonary bypass (CPB).

4.2. MATERIALS AND METHODS

4.2.1. Study design

Between the period from January 2010 to July 2010 in Department of Cardiac Surgery, Zala County Hospital and from April 2010 to September 2010 in Heart Institute, University of Pécs patient subjected to open heart surgery were prospectively enrolled in the study. Exclusion criteria before enrolment were the following: perioperative MI, impaired left ventricular function, ejection fraction less than 40%, unstable angina, recent or occurring AMI (in 6 month), preoperative ischemia, acute heart failure, left ventricular end diastolic dimension (LVESD)>40mm, significant hypertrophy (septal, posterior wall diameter>15mm, left ventricular mass index>130g), and renal insufficiency. Total of 758 patients were enrolled for further assessment. Patients underwent first time elective CABG surgery on CPB. Eight and twenty four hours after surgery blood samples were taken for assessment of plasma myocardial based creatinin kinase (CKMB) and troponin I (TI) levels. Cardiac markers were measured as a part of the routinely used clinical laboratory investigations, namely TI level was measured by LIASON CLIA chemiluminescence method (DiaSorin, Italy) and CKMB level was assessed by using MODULAR P800 analysator and commercially available kit

(Roche, Germany). Normal range of CKMB was determined as lower than 25 U/l for CKMB and 0.03 ng/ml for TI. Patients were continuously monitored for ECG and patients with highly elevated TI and/or CKMB levels and ECG changes of ischemia and/or haemodynamic instability undergone coronarography. Patients with postoperative renal insufficiency, prolonged intubation, hypoxia, prolonged postoperative hypotension, excessive bleeding, ECG abnormalities referring regional ischemia, postoperative regional wall motion abnormality on echocardiography, graft occlusion and assessed by coronarography and those patients who undergone an operation with prolonged aortic cross clamp and extracorporeal circulation (90 and 120 min.), or receive more than 2 units of RBC transfusion, were excluded from study.

Patients were divided into two groups: Group I: control patients (n=78) without any evidence of AMI in the postoperative period, those patients had less than 2.5 times elevation of CKMB or TI level of of upper limit of reference range as assessed by any of blood samples during the first postoperative 24 hours. Group II.: PMI patients (n=54) more than 5 times elevation of cardiac markers in 24 h following surgery, without any evidence of regional ischemia, or prolonged ischemia during surgery as described above in exclusion criteria.

Blood samples were taken from both groups for genotype analysis. Ultimately 132 patients (89 male and 43 female, mean age 59,84 years) were selected for the trial. Clinical characteristics of patients are presented on **Table 2**.

The main clinical features and general laboratory data of participants with PMI and control subjects are shown in **Table 2** presented as mean ± standard error of the mean (SEM).* $p<0.05$

	Control	**PMI**	**p value**
Age (years)	60,54±1,14	59,5±1,78	0,35
Gender (female %)	29	37	0,37
EuroSCORE I	5,77±0,28	5±0,36	0,13
Plasma levels of CK-MB (U/l)	50,79±2,63	141,88±9,04	$8.4 * 10^{-4}$
Plasma levels of troponin I (ng/ml)	0.26 ± 0.05	1.22 ± 0.29	0.0012
Preoperative ejection fraction	51,89±0,83	53,59±0,8	0,21
Postoperative bleeding (ml)	363,43±18,47	396±29,56	0,39
Aorta cross clamp time (min.)	50,29±2,13	54,38±1,86	0,2
Cardiopulmonary bypass (CPB) time	79,10±2,81	86, 4±3,03	0,12

4.2.2. Genotype analysis

The molecular analyses were performed using DNA extracted from peripheral EDTA-anticoagulated blood leukocytes with a routine salting out procedure. TaqMan SNP genotyping assay procedure were employed to analyze the GSTP1 gene region (rs2370143). The investigated chromosome location can be found at Chr.11: 67351774. The TaqMan Copy Number Assay (CNV) details were as follows: the chromosome location was Chr.11:67352208, the cytoband of GSTP1 can be located at 11q13.2b position. Taqman Assays was used with standard PCR conditions. (Life Technologies Cat Number: 4351379) The context sequence was:

TTTACCCCGGGCCTCCTTCCTGTT[T/C]CCCGCCTCTCCCGCCATGCCTGCTC.

TaqMan CNV genotyping assay procedure were performed to investigate the GSTM1 and GSTT1 regions. The analized chromosome location can be found at Chr.1:110231516 for GSTM1 and Chr.22:24376493 for GSTT1. The cytobands of GSTM1 and GSTT1 can be located at 1p13.3b and 22q11.23b positions. The amplication lengths were 100 and 82 nucleotids. (Life Technologies Cat. Numbers for GSTM1 and GSTT1: 4400291) For the molecular analyses, MJ Research PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA) was used. The two-step PCR conditions were as follows: predenaturation at 96 °C for 10 min; followed by 40 cycles of denaturation at 95°C for 20 s for all variants, annealing, primer extension and final extension at 60°C for 40 s for all the polymorphisms. (Lifetechnologies Applied Biosystems International, Incorporated.)

54 patients with AMI after open heart surgery and 78 patients without AMI after the open heart surgery were genotyped for polymorphism in the GST isoform P1, M1 and T1. In the case of GSTP1 there are 3 polymorphic GSTP1 alleles, GSTP1*A, GSTP1*B, and GSTP1*C expressed in normal cells. The variant DNAs result from A to G and C to T transitions at nucleotides 313 and 341, respectively. The transitions changed codon 105 from ATC (ile) in GSTP1*A to GTC (val) in GSTP1*B and GSTP1*C, and changed codon 114 from GCG (ala) to GTG (val) in GSTP1*C. Both amino acid changes are in the electrophile-binding active site of the GSTP1 peptide.

In contrast the GSTM1 and GSTT1 null phenotype appears to be caused by the deletion (two null alleles; -/-) of the GSTM1 or GSTT1 genes. Gene deletion in only one allele (+/-) also causes enzyme activity to decrease, so we could assume that this deletion also could be

involved in PMI. We are talking about full enzyme activity in the case when both of the alleles are consummate (+/+).

The presence of genetic polymorphism that decrease or absence activity of the detoxification enzyme GST may contribute to heart disease and affect biomarkers of coronary health and oxidative stress.

4.2.3. Statistical analysis

All clinical data are presented as mean ± standard error of the mean (SEM) where appropriate. Differences between the clinical parameters in patients with PMI and the control subjects were assessed with chi square (χ^2) test or the Mann-Whitney tests. Frequencies of different genotypes in PMI versus control groups were analyzed by χ^2 test. Odds ratios (ORs) originated from multiple logistic regression were used to determine whether the presence of the significant genotypes of the GSTP1-related variants were associated with the risk of the occurrence of PMI. The 95% confidence intervals (95% CI) for all ORs were calculated with SPSS 20.0 package for Windows (SPSS Inc, Chicago, IL, USA) and were considered significant when P-value was less than 0.05.

4.3. RESULTS

Table 2 shows the perioperative characteristics of patient groups. There was no significant difference between groups regarding gender and age, preoperative risk factors and postoperative bleeding. Intraoperative factors such as aorta cross clamp time (ACCT), and CPB times, operation time were not significantly different between groups.

However, there was no statistically significant difference in the changes of ejection fraction between the patient and control groups, tendency is well-marked, namely the ejection fraction is higher in the control group 4-6 days after the surgery compared to PMI group. (data not shown).

4.3.1 Genotype analysis of GSTP1

As seen in **Table 3.** there was no difference in the allele frequency of homozygous AA and the heterozygous AB and BC alleles between the groups, while it was almost three times elevated heterozygous AC allele combination observed in the patients who suffered PMI. There was 14% allele frequency of homozygous BB allele combination measured in the control group meanwhile in the PMI group BB allele combination was not present.

Comparing the two groups for CC homozygous allele combination, in controls 1.3% allele frequency was found, however, there was no such occurence amongst patients. From statistical point of view this data is not relevant (controls n=1 vs. PMI n=0), because of its low presence, which may be characteristic for Hungarian population.

We could not find any significance in disease risk in the carriers of the allele combinations with multiple regression analysis for age and gender. (**Table 4**)

It can be stated that in the presence of the A allele there is a significant difference between groups (OR (non adjusted): 12.614 (1.613–98.631); p = 0.016). This association is more markedly after adjusting the multiple regression analysis for age and gender (OR adjusted for differences in age and gender: 14.905 (1.859–119.495); p = 0.011). The presence of A allel is presented as a risk factor. However we failed to evidence the statistically significant protective effect when B allel is present (OR adjusted for differences in age and gender: 0.610 (0.293–1.269); p = 0.186). **(Table 5-6.)** Therefore it is possible that the A allele may have predisposing factor to PMI.

Table 3 Genotype frequencies in PMIs and controls.

PMI group (n=54)			
	A	**B**	**C**
A	26 (48.1%)	17 (31.5%)	10 (18.5%)
B	-	-	1 (1.9%)
C	-	-	-
control group (n=78)			
	A	**B**	**C**
A	37 (47.4%)	20 (25.6%)	6 (7.7%)
B	-	11 (14.1%)	3 (3.8%)
C	-	-	1 (1.3%)

Table 4 Multiple logistic regression analysis of disease risk of investigated variants with allele frequencies.

	OR frequency		
	A	**B**	**C**
A	1.029 (0.514-2.061) p=0.936	1.332 (0.619-2.869) p=0.463	2.727 (0.927-8.026) p=0.068
B	-	-	0.473 (0.048-4.664) p=0.521
C	-	-	-

P<0.05 versus controls

The 95% confidence intervals (95% CI) for all ORs were calculated.

Table 5. Multiple logistic regression analysis of disease risk of investigated variants from the perspective of haplotype carriers.

Not adjusted OR frequency		
A	**B**	**C**
(AA+AB+AC)	**(AB+BB+BC)**	**(AC+BC+CC)**
12.614*	0.647	1.740
(1.613-98.631)	(0.315-1.331)	(0.681-4.443)
p=0.016	p=0.237	p=0.247

P<0.05 versus controls

The 95% confidence intervals (95% CI) for all ORs were calculated.

Table 6 Multiple logistic regression analysis of disease risk of investigated variants from the perspective of haplotype carriers.

Adjusted OR* frequency		
A	**B**	**C**
(AA+AB+AC)	**(AB+BB+BC)**	**(AC+BC+CC)**
14.905*	0.610	1.755
(1.859-119.495)	(0.293-1.269)	(0.681-4.520)
p= 0.011	p=0.186	p=0.244

P<0.05 versus controls

OR*- Adjusted for differences in age and gender.

The 95% confidence intervals (95% CI) for all ORs were calculated.

4.3.2 Genotype analysis of GSTM1

The analysis for GSTM1 gene showed that in the PMI group 8 of 54 samples (14,8%) analyzed showed the null genotype, whereas the remaining 85.2% (46 of 54) carry one or two copies of the allele. In the control group was the same, namely 7 of 78 samples (9,0%) carried the null genotype and 71 of 78 (91,0%) have one or two copies of the allele. (**Table 7**)

Using a statistical method, we found no significant correlation between the groups (P = 0.303), as shown in **Table 8**.

None of the obtained frequencies for the GSTM1 gene polymorphisms studied showed significant differences with data reported for the same ethnic population.

Table 7 Genotype frequencies of GSTM1 gene.

	PMI group n=54	**Control group n=78**
	GSTM1	**GSTM1**
0	8 (14.8%)	7 (9.0%)
1	11 (20.4%)	20 (25.6%)
2	35 (64.8%)	51 (65.4%)
0 genotype %	25%	21,79%

Table 8 Multiple logistic regression analysis of disease risk of GSTM1.

Not adjusted OR frequency		**Adjusted OR* frequency**	
GSTM1		**GSTM1**	
1.764	(0.599-5.195)	1.728	(0.580-5.149)
p=0.303		p=0.326	

P<0.05 versus controls

The 95% confidence intervals (95% CI) for all ORs were calculated.

4.3.3 Genotype analysis of GSTT1

Of 78 control patients, 21 (26.9%) were homozygous for the GSTT1 null allele (**Table 9**). In contrast, 57 cases of the samples carrying the gene in hetero- or homozygosity (73,1 %) showed a stable GSTT1 genome (without detection of mutations or instability). In the PMI group this rate was 6 of the 54 patients (11.1%), while the hetero- or homozygous carrying was 46 patient (85,2%) and there was 2 (3,7%) missing data, where our measurement was

unsuccessful. This opposite values of observed percentages indicate that the GSTT1 null genotype may be correlated with the absence of PMI occurrence after cardiac surgery. Evaluating this possibility with statistical methods, we observed that, effectively, there was a positive significant association (P=0,039) for the events mentioned above (**Table 10**). Moreover, the OR value obtained (OR = 0,354 [0,132–0,950]; 95% CI), confirms the correlation between the homozygous deletion of the gene as a protective factor for PMI incidence.

TABLE 9 Genotype frequencies of GSTT1 gene.

	PMI group n=54	**Control group n=78**
	GSTT1	**GSTT1**
0	6 (11.1%)	21 (26.9%)
1	3 (5.6%)	1 (1.3%)
2	43 (79.6%)	56 (71.8%)
	missing:2 (3.7%)	
0 Genotype%	14.42%*	27,56%

Table 10 Multiple logistic regression analysis of disease risk of GSTT1.

	Not adjusted OR frequency	**Adjusted OR* frequency**
	GSTT1	**GSTT1**
	0.354* (0.132-0.950) p=0.039	0.317* (0.116-0.869) p=0.026

P<0.05 versus controls

The 95% confidence intervals (95% CI) for all ORs were calculated.

4.4. DISCUSSION

PMI is one of the most frequent and important complication of cardiac surgery and its clinical risk factors are well described. It was also determined that 30 day and two year survival is markedly decreased, furthermore hospital stay and ventilation time is increased following PMI[133]. Further impressing finding of recent studies describes that PMI appears irrespective of occurrence of graft occlusion. The relative high inter-patient variance in occurrence of PMI independently of described risk factors may suggest the role of genetic predisposition. In our present study, for better differentiation between PMI and control groups, the patients with well known clinical risk factors for PMI were excluded thus conducting the further, genetic investigation in 132 patients amoung 750 consecutive, patients undergoing elective, first time cardiac surgery enrolled in our study.

Recent papers stated the association of genotype variation and incidence of complication following cardiac surgery[134135136137138]. Eifert and coworkers described that genetic polymorphism of apoplipoproteins and nitric oxide synthetase permitted increased reintervetion rate and thus limited prognosis in patients receiving coronary surgery [139]. Other study highlighted the role of b receptor/ G protein polymorphism in development of adverse outcome after CABG[140]. Further evidences highlighted the role of polymorphism of E selectin gene in PMI following CABG via activation of platelets[141]. Kuang-Yu Liu and co-authors revealed association between given genetic variants in 9p21 and PMI following on-pump CABG. Further study stated increased mortality in patients undergoing CABG in presence of 9p21 variant rs10116277 gene[142]. On the other hand the study of Virani et al however failed to find any association between chromosome 9p21 SNPs and reoperation, recurrent myocardial infarction rate following CABG[143]. Recently, Podgoreanu et al. have reported that candidate gene polymorphisms in inflammatory pathways contribute to risk of PMI after cardiac surgery. Other studies investigated the role of polymorphisms of different genes in inflammatory response following cardiac surgery which may be associated with perioperative myocardial injury [144145146147]. Our findings indicate the possible role of presence of GSTP1 A haplotype in the pathogenesis of PMI.

The GSTs belong the most important antioxidant enzymes that play an important role in detoxification conjugating toxic compounds with reduced GSH. Safe elimination of reactive electrophilic molecules via GST pathways protected cells against mutagen and reactive oxygen induced damage [148]. The mammalian cytosolic GSTs are divided into seven classes,

these are alpha, mu, kappa, theta, pi, omega and zeta. The GSTP1 is one of the most investigated isoenzyme which is widely expressed in different human epithelial tissues. Individuals presented with genetic polymorphism of GSTP1 have different enzymatic activity [149] altering detoxification activities. A null allele at the GSTM1 locus has a high frequency of about 0.7, however the null phenotype has a frequency of greater than 50% among Caucasian, Chinese, and Indian populations. GSTT1 has been implicated mostly in detoxifying mutagenic electrophilic compounds.

Inter-individual differences in response to xenobiotics and oxidative stress products resulting from polymorphism have been grown to the groundwork for studies of the role of this gene in xenobiotic metabolism, cancer, cardiovascular and respiratory diseases [150151]. Our finding is consistent with several studies in relation to the role of GST polymorphism on the development of cardiovascular disease. Christie et al. and Hadjiliadis et al. proved an association between different donor GST genotypes and primary graft dysfunction in patients underwent heart and lung transplantation[152153].

Since its primary function of GST in detoxification the polymorphism of enzyme is well investigated in tumorogenesis and resistance against chemotherapy. Moscow et al. found that GSTP1 expression was significantly increased in many tumors relative to matched normal tissue[154]. Allan et al. suggested that polymorphisms in GST genes alter susceptibility to chemotherapy-induced carcinogenesis[155]. Tuna et al. highlighted that inhibition of GSTA and GSTP1 by hypericin might increase the effectivity of the chemotherapy treatment[156].

Henderson et al. described that GSTP1 carries conjugation of acetaminophen resulting more toxic compound thus in GSTP1 null mice were highly resistant to the hepatotoxic effects during administration of acetaminophen[157].

PMI as a complication of cardiac surgery is associated with significant mortality and morbidity. Besides of classic, well-known risk factors of PMI, such as prolonged CPB or ACCT, longer surgery duration, preoperative, ongoing myocardial ischemia and inadequate revascularization, graft occlusion, unstable angina, emergent operation, other factors and genetic predisposition are expected to postoperative myocardial damage. In the present study we investigated the genetic contribution of GSTP1, GSTM1 and GSTT1 to occurrence of PMI. Our results suggest that the genetic polymorphism of GSTP1 is independently associated with the presence of PMI. The appearance of homozygous BB allele combination was significantly higher in the control group. In present paper it is suggested that the existence of the A allele carries complication for PMI. Although in the present report we mentioned that GSTM1 null genotype could be a risk factor for PMI presence, our results

have shown that the GSTM1 null genotype (P = 0.303) have not significant association with the presence of PMI in CPB patients (Table 8). A possible explanation is that, although expression of these genes was detected in the mammary tissue, the encoded enzymes only weakly expressed in cardiac muscle. This allows us to conclude that the action of metabolites is not mediated and/or regulated by this enzyme on a local level. So, the presence or absence of GSTM1 in PMI patients was independent from each other. In contrast in other studies it was also confirmed that GSTM1 null genotype is protective against coronary artery disease[158 159160161]. Our results have identified that GSTT1 null genotype play a protective role against PMI (OR:0.354, p=0.039). However we can assume that the effect of GSTT1 against PMI is secondary because in humans, the GSTT1 enzyme is found just in the erythrocyte, in this way it plays a smaller role in the struggle for survival of cardiomyocytes.

The major limitations of this clinical part are the relatively small sample size which limits significant conclusions. The size of groups might be increased to improve the statistical power of the study. On the other hand it would be advantageous to follow up the patient in longer term for assessing the late complications associated with GST polymorphisms. Furthermore the enzymatic activity of GST was not measured in association of polymorphisms in groups.
In conclusion, genetic variants in GSTP1 are independently associated with an increased risk of PMI after surgical coronary revascularization. Our results highlight the potential genetic role in etiology of PMI following cardiac surgery. To the best of our knowledge, this is the first study that has investigated the role of GST polymorphisms with PMI and we found GSTP1 A haplotype as a predisposing factor and identified BB homozygous allele combination as a possible protective factor with PMI and besides GSTT1 null genotype also may act a protective role with PMI.
Further large scale genetic association and molecular studies are required to delineate the clinical implications and mechanisms underlying these observations.

5. NOVEL FINDINGS

1) Firstly we demonstrated that the pharmacological inhibition of GST could markedly exaggerate oxidative stress parameters and induced apoptosis in vivo rat model. Measuring of total infarct size we were capable to detect that the infarction size was significantly higher in the presence of EA while PC limited the extent of infarcted area. We were able to confirm that presence of EA is capable to eliminate the protective effect of PC.

2) GST inhibition was associated with different activation of MAPK and RISK kinases regulating these pathways under myocardial stress conditions. We detected, that GST plays crucial role among pro- and antiapoptotic MAPK and RISK kinases in the process of oxidative stress-induced apoptosis in an in vivo rat model.

3) We were able to demonstrate that GST inhibition could markedly attenuate the protective effect of ischaemic PC and resulted in increasing apoptosis in in vivo rats during myocardial ischemia. It was clarified that in the process of PC GST inhibition is in the close association with activation of different MAP kinases and RISK protein kinases.

The proapoptotic p38 and the GSK-3β activation was decreased in PC group conversely in the presence of EA this protective effect was strongly reduced. Ischaemic postconditioning could significantly enhance the antiapoptotic ERK and the phospho-Bad activity in PC group but in case of GST inhibition we could not detect this protective effect.

4) Our results propose that the genetic polymorphism of GSTP1 is independently associated with the presence of PMI. The appearance of homozygous BB allele combination was significantly higher in the group without complications (non-periop AMI group). In present paper it is suggested that the existence of the A allele carries complication for PMI.

As yet, there have been no previous analyses examining the association of GSTP1, GSTM1 and GSTT1 polymorphisms with the development of PMI. Our prior findings of genetic data from GSTM1 studies and PMI suggested that the null genotypes at GSTM1 had no effect on PMI.

Our results have identified that GSTT1 null genotype play a protective role against PMI. Our results highlight that polymorphisms could play predisposing role in progression of PMI following cardiac surgery.

New findings will enable us to have a better understanding about the mechanism of interrelationships which converge in the event of genomic variants, as well as for the real-time enzymatic processes themselves.

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7. PUBLICATIONS AND PRESENTATIONS

7.1 PUBLICATIONS RELATED TO THE THESIS

1. Z. Miklós, M. Kürthy, P. Degrell, E. Ranczinger, M. Vida, J. Lantos, E. Arató, L. Sínay, P. Hardy, B. Balatonyi, S. Ferencz, Sz. Jávor, **V. Kovács,** B. Borsiczky, Gy. Wéber, E. Rőth, G. Jancsó. Ischaemic postconditioning reduces serum and tubular TNF-alpha expression in ischaemic-reperfused kidney in healthy rats, Clinical Hemorheology and Microcirculation 47 (2011) 1-12; DOI 10.3233/CH-2011-1414, 2011**. IF: 3.40**

2. E. Rőth, N. Marczin, B. Balatonyi, S. Ghosh, **V. Kovács**, N. Alotti, B. Borsiczky, B. Gasz. Effect of glutathione S-transferase inhibitor on oxidative stress and ischaemia-reperfusion-induced apoptotic signalling of cultured cardiomyocytes.
   Experimental & Clinical Cardiology Vol. 16; No 3; 92-96; 2011. **IF: 0,576**

3. Balatonyi Borbála, Gasz Balázs, Subhamay Ghosh, Lantos János, **Kovács Viktória**, Jávor Szaniszló, Wéber György, Rőth Erzsébet, Marczin Nándor. A glutation S-transzferáz gátlásának hatása oxidatív stresszel károsított szívizomsejtekre.
   Cardiológia Hungarica 2011; 41; No 5 : 319-324

4. B. Balatonyi, B. Gasz, **V. Kovács**, J. Lantos, G. Jancsó, N. Marczin, E. Rőth The role of the inhibition of glutathione S-transferase /GST/ in the protective mechanisms of ischemic postconditioning.
   Canadian Journal of Physiology and Pharmacology-10.1139/cjpp-2012-0411 **IF: 1.546**

5. Borbála Balatonyi, Balázs Gasz, **Viktória Kovács**, János Lantos, Gábor Jancsó, Nándor Marczin, Erzsébet Rőth- Influence of MAPK Inhibitors on the Oxidative Stress of Isolated Cardiomyocytes- Journal of Proactive Medicine Vol 2. No 1. (2013)

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7.4 PRESENTATIONS

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