

The role of the endogenous antioxidant enzyme, glutathione S-transferase on cultured cardiomyocytes under oxidative stress conditions

PhD Thesis

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ABBREVIATIONS

Akt	Protein kinase B
ASK1	Apoptotic signal-regulating kinase-1
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CSFM	Complete serum free medium
Cyt C	Cytochrome C
DMEM/F-12	Dulbecco's Modified Eagle' Medium medium/Ham's nutrient mixture F12
DMSO	Dimethylsulfoxide
EA	Ethacrynic acid
EDTA	Ethylene-diamin tetra-acetate
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S transferase
GSK 3β	Glycogen synthase kinase-3 β
H₂O₂	Hydrogen-peroxide
IKK	Inhibitor of nuclear factor kappa-B kinase
IPoC	Ischaemic postconditioning
I/R	Ischaemia/reperfusion
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MFI	Mean fluorescence intensity
mPTP	Mitochondrial permeability transition pore
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NFκB	Nuclear factor kappa-B

NO	Nitric oxide
p38	p38 mitogen activating protein kinase
PI	Propidium iodide
PBS	Phosphate buffered saline
PI 3 kinase	Phosphatidylinositol triphosphate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear cell
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1. INTRODUCTION

Myocardial ischemia-reperfusion injury is crucially involved in the pathogenesis of cardiovascular diseases. It has been well investigated that oxidative stress following ischaemic-reperfusion injury is a major apoptotic stimulus in many cardiac diseases. Under normal circumstances the endogenous antioxidant systems neutralise the harmful effects of free radicals. Pathophysiological conditions (such as hypoxia, ischemia, early reperfusion, etc) the amount of nascent oxygen free radicals and reactive oxygen intermediers (ROI) beyond the capacity of endogenous antioxidants and the oxidative stress develops.

Although recently surgical interventions more frequent in myocardial diseases, but to avoid the ischemic-reperfusion injury is not solved. The role of oxygen free radicals in reperfusion injury is well known, accordingly to decrease of these harmful agents is very important. Catalase, superoxide dismutase (SOD), glutathione peroxidase and repair enzymes are in the first line of antioxidant protection, but recently among other antioxidant enzymes researches are focus on *glutathione S-transferase (GST)*.

Myocyte loss during ischemic-reperfusion injury involves both apoptotic and necrotic cell death. Therefore, it is reasonable to think that the balance of cell survival and death is critical during the pathological evolution of postischemic cardiac dysfunction. The intricate relationship between signal transduction and this balance of survival and death in oxidative stress and ischemic-reperfusion injury make the investigations necessary to be focused on MAP kinases.

1.1. GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE

1.1.1. Glutathione

Glutathione (GSH) is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes) (1). With the exception of bile acid, which may contain up to 10 mmol/L GSH, extracellular concentrations of GSH are relatively low (e.g., 2–20 μ mol/L in plasma) (2,3). Because 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).

Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (1,3). The [GSH]:[GSSG] ratio, which is often used as an indicator of the cellular redox state, is >10 under normal physiological conditions (3). GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells, but its value can be affected by other redox couples, including NADPH/NADP⁺ and thioredoxin_{red}/thioredoxin_{ox} (2). It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, calcineurin, nuclear factor κ B, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase), thereby reducing cell proliferation and increasing apoptosis (4).

A major factor that affects glutathione homeostasis is its utilization by conjugation, primarily via glutathione S-transferase (GST) (5,6).

1.1.2. Glutathione S-transferase

Glutathione S-transferases (GSTs), are members of a multigene family of isoenzymes ubiquitously expressed in most living organisms. It was subsequently shown that these enzymes catalyze the conjugation of glutathione (GSH) to a variety of electrophilic compounds, thus establishing the now widely accepted role of GSTs as cell housekeepers involved in the detoxification of endogenous as well as exogenous substances (7,8).

The GSTs encompass three major families of proteins: (1) cytosolic, (2) mitochondrial, and (3) microsomal (also referred to as membrane-associated proteins in eicosanoid and glutathione /MAPEG/), of which the cytosolic GSTs constitute the largest family (9). On the basis of amino acid sequence similarities, substrate specificity, and immunological cross-reactivity, seven classes of cytosolic GSTs have been identified in mammals (10,11,12). These classes are designated by the names of the Greek letters α (alpha), μ (mu), π (pi), σ (sigma), θ (theta), ω (omega), and ζ (zeta) (13,14).

The ability of GST to alter levels of cellular glutathione in response to production of ROS has been implicated in protection of cells from ROS-inducing agents (15,16). Accumulation of ROS in response to UV or H₂O₂ treatment 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 (mitogen-activated protein kinase), (17,18), ERK/p42-44 (19), and IKK-NFκB (20,21) signaling pathways. Importantly, the balance between different stress signaling cascades appears to be among the key determinants in dictating the cell's fate (22,23), although the mechanisms underlying the coordinated regulation of the kinases involved are not understood (24).

More recently, isoenzymes from several GST classes have been shown to associate with members of the mitogen activated protein kinase (MAPK) pathways involved in cell survival and death signaling. In this non-enzymatic role, 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 I/R injury, cell proliferation and apoptotic cell death (25).

1.2. ISCHAEMIC/REPRFUSION INJURY

1.2.1. General background

Myocardial reperfusion is the restoration of blood flow to an ischemic heart. Early reperfusion minimizes the extent of damage of heart muscle and preserves the pumping function of the heart. However, reperfusion has been referred as a „double edged sword” (26) because after a prolonged period of ischemia reperfusion produces a marked damage in myocardium rather than restoration of normal cardiac function. This reoxygenation injury is mediated by a burst of ROS production. Thus, ischemia–reperfusion (I/R) injury could be defined as the damage to heart when blood supply is restored after a prolonged period of ischemia resulting in oxidative damage, inflammation and cardiac dysfunction (27). Reperfusion injury is an integrated response to the restoration of blood flow after ischaemia involving mechanical, extracellular and intracellular processes. It is initiated at the very early moments of reperfusion, lasting potentially for days. Some of the events that trigger reperfusion injury are:

- Rapid generation of reactive oxygen species (ROS) by activated vascular endothelial cells, neutrophils (NADPH oxidase, myeloperoxidase-MPO), lipid mediators (platelet activating factor-PAF, leukotriene B₄-LTB₄, polipeptide mediators-C_{5A}) and stressed cardiomyocytes (28).

- Activation of sodium-hydrogen exchanger (29) and augmentation of ischemia induced cellular and mitochondrial Ca^{2+} overload (30).
- Increased osmotic gradient and cell swelling induced by the accumulation of products of anaerobic metabolism (31).
- 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 (32).
- Reduced NO availability (33) leading to the augmented expression of cellular adhesion molecules, induction of local inflammation, leukocyte infiltration and no-reflow phenomenon.

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.

1.2.2. Apoptosis in ischaemic/reperfusion injury

Apoptosis, a physiological process for killing cells, is one of the main types of programmed cell death which occurs during development, aging and several other processes. The balance and the ratio of apoptosis is very important, because abnormalities in cell death control can contribute to a variety of diseases for example too much apoptosis causes cell-loss disorders, whereas too little results in uncontrolled cell proliferation, namely cancer or autoimmune disorders. This balancing process is part of the homeostasis. Homeostasis is achieved when the rate of cell proliferation in the tissue is balanced by cell death.

The level of apoptosis was shown to depend on the duration of reperfusion (34). Ischemia by itself can trigger apoptosis, reperfusion accelerates the process.

Signaling for apoptosis occurs through multiple independent pathways that are initiated either from triggering events within the cell or from outside the cell. Apoptosis depends on the activation of a family of proteinases with essential active-site cysteine residues, the caspases. Caspase activation can be triggered by at least four mechanisms:

- Binding of external ligands to death receptors.

- Damage of mitochondria. – This can lead to permeabilization of the outer mitochondrial membrane and release of factors through the mitochondrial permeability transition pore (mPTP) from the intermembrane space to the cytosol, including cytochrome c (Cyt C) and other pro-apoptotic molecules like apoptosis inducing factor (AIF), and SMAC/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI).
- Introduce an agent that directly activates caspases and/or mitochondrial proapoptotic proteins.
- Endoplasmic reticulum (ER) stress response can also lead to apoptosis. (35)

The mitogen activated protein kinase (MAPK) cascades, are generally activated in response to mitogenic signals, cytokines and environmental stress. Three subfamilies have been identified in mammalian cells: extracellular signal-regulated kinase-p44/42 (ERK/p42-44), p38 MAPK (p38) and c-Jun N-terminal kinase (JNK). These MAPK cascades have been shown to participate in several cellular functions, such as cell proliferation, cell differentiation, cell motility, apoptosis and usually lead to alterations in transcription factor activity to modulate gene expression.

MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli.

Although many MAPK activating stimuli are 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/p42-44 pathways are usually linked to growth factor action and are associated with cellular differentiation, proliferation and tends to promote cell survival. JNK and p38 MAPK are serine and threonine protein kinases that are activated by various stress stimuli and favour cell death, for example by apoptosis. 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). Akt/PKB promotes cell survival, for example by phosphorylating and

decreasing the activity of proapoptotic factors such as Bad (Bcl-2-associated death promoter) and caspase-9 (36).

In 1999 Yellon and Baxter first introduced the concept of a pro-survival reperfusion signalling pathway which they termed the Reperfusion Injury Salvage Kinase (RISK) pathway (37). It has demonstrated that the activation of pro-survival kinases such as Akt/PKB, ERK/p42-44 and GSK-3 β (the RISK pathway) at the immediate onset of myocardial reperfusion reduces myocardial infarct size in the region of 40-50%. (38,39) One particular downstream target of the RISK pathway is glycogen synthase kinase-3 (GSK-3) a protein kinase is highly evolutionarily conserved and present in every eukaryotic species. GSK-3 is found in the cytosol, mitochondria, and nucleus of cells, (40) and approximately 50 substrates have been identified. GSK-3 activity has been associated with many cell processes, including the regulation of multiple transcription factors, nuclear factor κ B, endoplasmic reticulum (ER) stress, embryogenesis, apoptosis and cell survival, cell cycle progression, cell migration. GSK-3 is a serine/threonine protein kinase. Two mammalian isoforms of GSK-3, α and β , have been identified (41). mPTP opening is a crucial event in lethal reperfusion injury (42). Phosphorylation of GSK-3 β causes the inhibition of the enzyme itself. GSK-3 β inhibition prevents the opening of the mitochondrial permeability transition pore (mPTP). Inhibition of mPTP opening inhibits the release of cytochrome C from mitochondria, a mechanism preventing apoptotic cell death (43).

In our study we focused on the activation of members of mitogen activated protein kinase (MAPK) family: c-Jun N-terminal kinase (JNK), p38 MAPK, extracellular signal-regulated kinase (ERK/p42-44) and Akt/PKB and GSK-3 β protein kinase.

1.3. ISCHAEMIC POSTCONDITIONING

1.3.1. The meaning of ischaemic postconditioning

To protect the heart against ischaemia and reperfusion injury ischaemic preconditioning (IPC) is a well known strategy (44). However, unpredictability of clinical acute myocardial infarction precludes the application of preconditioning.

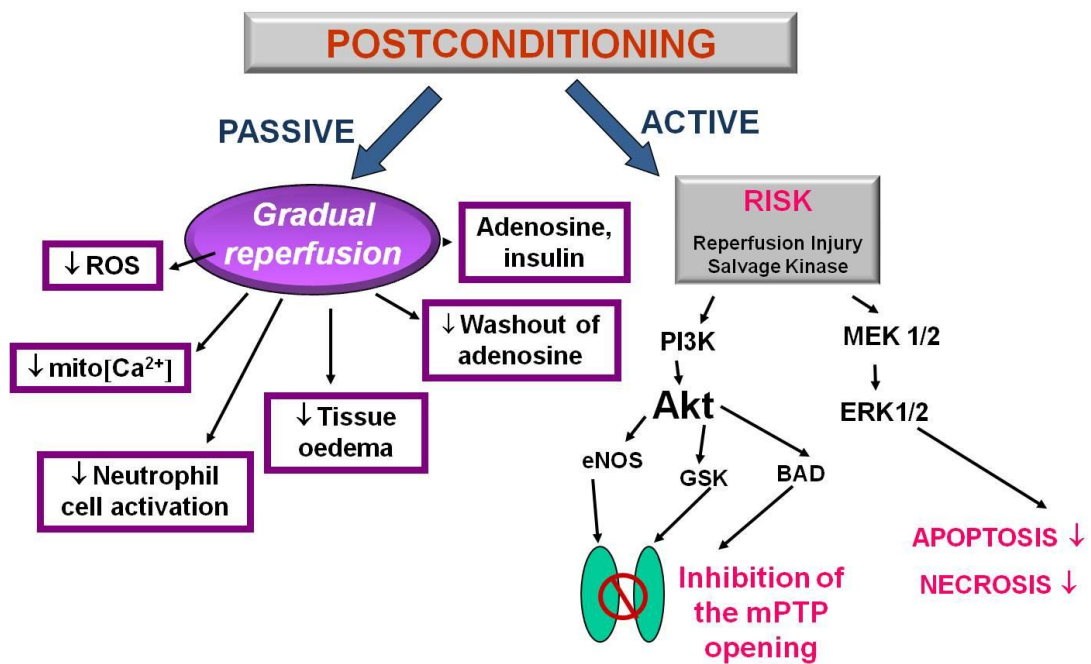
Postconditioning is controlled reperfusion (45), defined as a series of brief interruptions of ischemia/reperfusion applied at the very onset of reperfusion can protect

the myocardium from ischaemic/reperfusion injury. It may have greater clinical potential than preconditioning because of the usability in cases of unintended interventions (44,46). Heusch described ischemic postconditioning as “old wine in a new bottle”. The concept of postconditioning was first revealed in 2002 by Vinten-Johansen and colleagues. The first studies published by Zhao et al., (2002).

1.3.2. The possible mechanism of protection induced by ischaemic postconditioning

Any cardioprotective strategy applied at the time of reperfusion must provide protection against the known mediators of lethal reperfusion injury, which include cellular and mitochondrial calcium overload, a burst of oxidative stress, endothelial dysfunction, and reduced nitric oxide production. The protective effect of postconditioning may directly or additionally be related to beneficial antiinflammatory or antioxidant effects. The mechanisms of postconditioning are realised on passive and active way. During perfusion pressure water extravasation can increased and cause edema, which is aggravated by microvascular injury. According to the *passive* way in the course of gradual reperfusion IPoC delays the washout of adenosine, decreases extracellular levels and the accumulation of noxious metabolites which attenuates superoxide anion generation by activation of neutrophils and endothelial cells, and activates mitochondrial KATP channels via adenosinergic G protein-coupled receptor activation. Better endothelial function increases nitric oxide release by endothelial cells, which further attenuates superoxide anion levels and both neutrophil activation and adherence to the endothelial cells. Postconditioning decreases the intracellular buildup of oxidants and calcium in cardiomyocytes, which inhibits mitochondrial permeability transition pore (mPTP) opening, thereby inhibiting both apoptosis and necrosis. Regarding the *active* way possible to protect the reperfused myocardium by activating prosurvival kinase signaling pathways (reperfusion injury salvage kinase pathway) (47). Both pre- and postconditioning activate the same key pathways, which include phosphatidylinositol 3-kinase-Akt and extracellular signal-regulated kinase (ERK/p42-44) (47,48). Upstream may be activation of G-protein coupled receptors, and the many downstream events include key phosphorylations of endothelial nitric oxide synthase and inhibition of the apoptosis promoters. Protective pathways activated by postconditioning appear to converge on the mitochondria, in particular the mitochondrial permeability transition pore.

This opens during the first few minutes of reperfusion, in response to mitochondrial calcium overload, oxidative stress, and adenosine triphosphate depletion (43). Postconditioning (49) protect the heart through the inhibition of mitochondrial permeability transition pore opening. Taken together postconditioning influence a variety of endogenous mechanisms that operate at numerous levels and target a broad range of pathological mechanisms. (Figure 1.)



J Vinten-Johansen, D Yellon, L Opie. Circulation.2005.Oct..2085-2088.

Figure 1. Hypothetical scheme of the possible mechanisms of protection induced by ischaemic postconditioning are realised on passive and active way.

2. AIMS AND HYPOTHESIS

In the first part of our investigations we aimed to identify the biological role of GST in cardiomyocytes under oxidative stress conditions. Principally, our aim was to evaluate the effect of GST inhibition (using its potent inhibitor, ethacrynic acid [EA]) on cardiomyocyte apoptosis and on the alteration of proteins and MAP kinase pathways.

In the second part of our study the main objective was to identify the role of MAPKs (JNK, p38 and ERK/p42-44) on the viability and apoptosis of cardiomyocytes when cells are exposed to various stress components of ischaemia and reperfusion using parallel GST and MAPKs inhibitors.

Finally, in the third we targeted to investigate whether inhibition of GST (by its potent inhibitor ethacrynic acid) can abolish the cellular mechanisms and benefit of ischaemic postconditioning (IPoC) in vitro ischaemic/reperfusion injury by assessing the cell viability and apoptosis in rat cardiomyocyte culture in addition on alteration of activities of mitogen activated protein (MAP) kinase pathways.

3. THE ROLE OF GLUTATHIONE S-TRANSFERASE IN CELL VIABILITY AND SIGNAL PATHWAYS IN CARDIAC MYOCYTES UNDER OXIDATIVE STRESS CONDITIONS

3.1. INTRODUCTION

A large collection of experimental data support the presence of apoptosis in a variety of cardiovascular diseases. It has also been well investigated that oxidative stress is a major apoptotic stimulus in many cardiac diseases (50,51,52). Among numerous defence mechanisms against oxidative injury, glutathione S-transferase (GST) plays a crucial role. The GST family, which comprises a relatively high amount of total cytosolic protein, is responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates (53,54). Thus, glutathione homeostasis is essentially regulated by GST activity, and the glutathione redox status is critical for various biological events. Recently, novel roles for glutathione homeostasis and GST in signal transduction, gene expression, apoptosis, protein glutathionylation, nitric oxide metabolism and inflammation have been discussed (4,54,55).

It is important to note that alterations of cellular-reduced glutathione (GSH) metabolism and activity of GST can influence several signalling pathways (6,54). Certain types of GST play a key role in regulating mitogen-activated protein (MAP) kinase pathways involved in the cellular response to stress, apoptosis and proliferation (56,57), thus altering activity of apoptotic signal-regulating kinase-1 (ASK1) and influencing the decision regarding cell fate.

At least five of the human GST genes display functional polymorphisms. These polymorphisms are likely to contribute to interindividual differences in response to xenobiotics and clearance of oxidative stress products and, therefore, may determine susceptibility to various inflammatory pathologies including cancer, and cardiovascular and respiratory diseases (11,58). In addition, some GST polymorphisms have also been associated with increased risk of lung adenocarcinoma (58,59). Recent studies highlight the potentially unique roles of GST enzymes as crucial determinants of the development of ischemia-reperfusion (I/R). An association was found between different donor GST genotypes and primary graft dysfunction in patients following heart and lung transplantation (60,61). Other studies described the damaging effect of GST inhibition on

peripheral and central motor neurons, cerebral astrocytes, isolated hepatocytes and vascular smooth muscle cells (62,63,64,65). Although the effect of GSH depletion in cardiomyocytes has been well described to be a result of different pathological states (54,66,67), the exact role of GST activity on cardiomyocyte apoptosis and alteration of signalling cascades of cardiomyocytes has not been determined.

3.2. AIMS

In present study we aimed to investigate the biological role of GST in cardiac myocytes under different stress conditions. Therefore we tried to identify the effect of GST inhibition via administration of EA when cells were exposed to various stress components of I/R. Since GST activity is a major determinant for survival and adaptive response to oxidative stress in the heart, thus we hypothesized that its pharmacologic inhibition with EA might exacerbate the severity and outcome of I/R and oxidative injury.

3.3. MATERIALS AND METHODS

The present study conforms with 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).

3.3.1. Isolation of cardiac cells from neonatal rats

Primary culture of neonatal rat cardiomyocytes was prepared as described previously (68,69). Cells were obtained from ventricular myocytes of 2-4 day-old Wistar rat pups (Charles-River Ltd., Hungary), using collagenase (Gibco™ Collagenase Type II, Invitrogen Corp., Carlsbad, CA, USA).

After decapitating the pups, we immersed briefly the carcasses in 70-96% ethanol to disinfect and moved them on a sterile Petri dish in a laminar hood. With sterile instrument set we removed both forelegs and skin covering the chest and to reveal the heart removed the chest with second set of sterile instruments avoiding damage of the heart. While the aorta was clamped with forceps, we perforated the right atrium with injection needle to

perfuse the heart with disaggregation medium I. – consisting Collagenase II (GIBCO) in PBS; 51 mM CaCl₂ by puncturing the apex of the ventricle. To get pure atrial and ventricular cell culture we removed the auricular appendages and apex of the heart and transferred them on separate glass Petri dishes containing disaggregation medium II (disaggregation medium I and PBS in ratio of 1:1).

After dissecting all the pups we cut the tissues into 1-2 mm fragments and transferred the minces to conical flasks and incubated at 37 °C with shaking.

After making the filtering units to 25 mm filter holder containing 100 µm nylon mesh (BD Biosciences-Cell Strainer) attached on Falcon tube (BD Falcon). Then we filtered the supernatants and added culture medium I – consisting of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (GIBCO), 100 IU/ml penicillin, 0,1 mg/ml streptomycin (GIBCO), 1,28% 200mM L-Glut (GIBCO) supplemented with 10 % fetal bovine serum (FBS, GIBCO) – onto filtrates. To the tissue minces we added fresh disaggregation medium I., mixed them, and we repeated the cycle of incubation and collecting the supernatant until all the tissues are disintegrated.

Then the filtrates were washed by centrifugation at room temperature, medium was discarded and the ventricular cells were resuspended in DMEM/F-12 10% FBS medium. After the second centrifugation the suspension was seeded on 100 mm cell culture dishes and incubated for 45 minutes in cell incubator (95 % air, 5% CO₂, 37 °C) to reduce the number of contaminating non-muscle cells. After gentle shaking, we collected the muscle cell-enriched suspension in cell culture bottle and counted the cells in Bürker hemocytometer taking 180 µl suspension and 20 µl trypan blue vital staining.

Thereafter the muscle cell-enriched fraction was seeded onto collagen I-coated 12-well plates (BD Falcon) at the density of 200 000 cells per ml and incubated in cell incubator. The cells were cultured in DMEM/F-12 medium (Sigma–Aldrich, USA) supplemented with 10 % of fetal bovine serum (Gibco, USA) for 24 hours (h). The following day, when the cells attached to the plate firmly, the medium was replaced with complete serum free medium (CSFM) – containing 2,5 mg/ml bovine serum albumin (BSA, AlbuMax 1, Invitrogen); 1 µM insulin; 5,64 µg/ml transferrin; 32nM selenium (insulin-transferrin-selenium media supplement, Sigma, Hungary); 2,8 mM Na-pyruvate (Sigma); 0,1-1 nM 3,3',5'-triiodo L-thyronine sodium salt (T₃, Sigma, Hungary); 100 IU/ml penicillin; 0,1 mg/ml streptomycin (PS solution, Sigma, Hungary); 200 mM L-Glut; DMEM/F-12 – to prevent the proliferation of non-muscle cells and to standardise the experimental

protocol. Within 2 days a confluent monolayer of spontaneously beating cardiomyocytes was prepared.

Experiments started 24 hours after incubation with CSFM and the medium was changed every 24 hours.

3.3.2. Ethacrynic acid is a potent inhibitor of GST

Present study utilised EA for pharmacological inhibition of GST. EA has been shown to be a substrate of majority of GST isozymes furthermore nonenzymatic GSH conjugation of EA also exists. Moreover it was shown that EA-SG was an inhibitor of the GSTs due to its greater affinity for the enzymes, whereas EA itself inhibits GST through reversible covalent interactions (70). **(Figure 2.)**

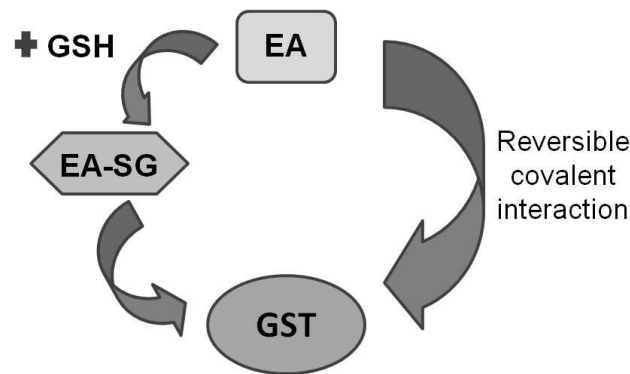


Figure 2. Ethacrynic acid (EA) inhibits GST in two ways.

3.3.3. Experimental protocol

Cultured cardiomyocytes were randomly assigned to one of six experimental groups: control group of cells that were incubated in CSFM without treatment (group 1). In the second group isolated cells treated with 150 μ M of EA alone (group 2). In the third group cells exposed to 1 mM of H_2O_2 (group 3). In the fourth group cells treated with 1 mM of H_2O_2 together with 150 μ M of EA (group 4). In the fifth group cells exposed to I/R (group 5). In the last group cells exposed to I/R and 150 μ M of EA (group 6). **(Figure 3.)**

To mimic the ischaemic stimuli cell cultures were exposed to ischaemic buffer as described previously (71) (simulated ischemia (SI) buffer) that contained 137 mM NaCl, 3.5 mM KCl, 0.88 mM $CaCl_2 \cdot 2H_2O$, 0.51 mM $MgSO_4 \cdot 7H_2O$, 5.55 mM D-glucose, 4

HEPES, 2% FCS, 10 mM 2-deoxy-D-glucose and 20 mM DL-lactic acid (pH 6.2). During the ischaemic stress insult cardiac cell cultures were incubated in cell incubator in an atmosphere of 95 % air and 5% CO₂, on 37 °C.

In groups receiving simulated I/R cells were exposed to 1,5 hours of ischemia using SI buffer, followed by 2,5 hours of reperfusion using normal CSFM. In group VI. (cells were exposed to both simulated I/R and EA) both ischemic buffer and reperfusion medium (CSFM) contained 150 µM of EA.

Based on our pilot experiments we chose to use a concentration of 150 µM and a treatment time of 4 hours.

Cells were exposed to mentioned concentration of chemicals for 4 hours. MTT assay evaluation of cell survival was performed immediately after termination of treatments. Assessment of apoptotic signalling markers was also started after treatments until permeabilization, and samples were stored at -20 °C until further processing according to the protocol supplied by the manufacturer. Experiments were repeated six times in duplicate wells.

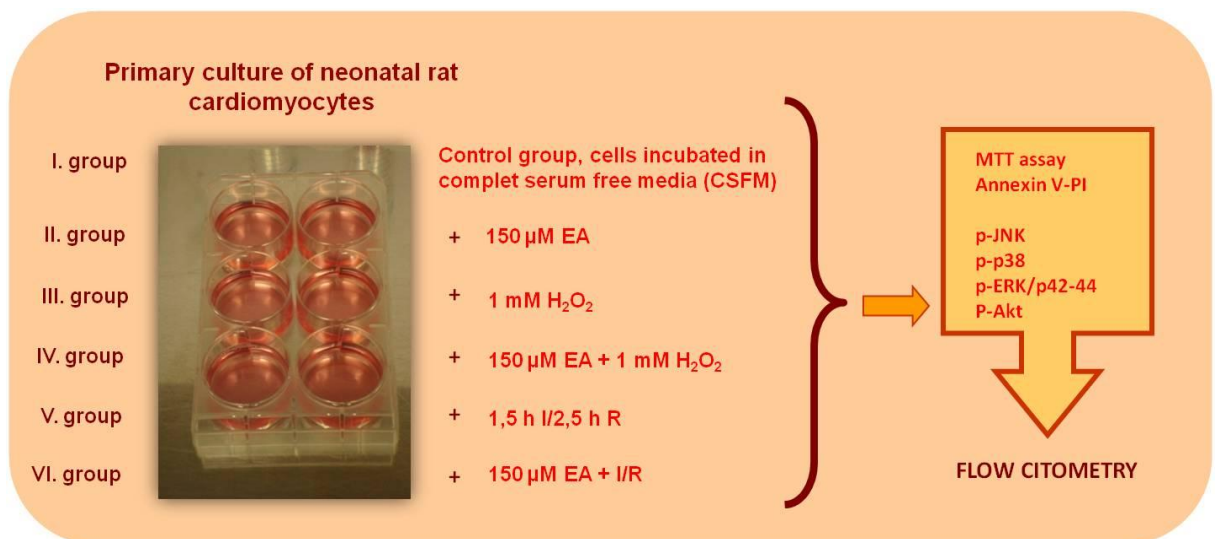


Figure 3. Experimental protocol, using GST inhibitor EA, oxidative stress (H₂O₂), simulated I/R and the combination of these. Viability of cells were measured by MTT assay, the amount of apoptotic cells were assessed by flow cytometry following annexin V-FITC/propidium iodide double staining. The activation of JNK, p38, ERK/p42-p44 and Akt MAPKs were determined by flow cytometric assay.

3.3.4. Cell viability test

Viability of cardiomyocytes was determined by colorimetric MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma]. The assay is based on the reduction of MTT into a blue formazan dye by the functional mitochondria of viable cells. The cells were seeded into 96-well plates at a density of 10^5 cell/well and cultured overnight before the experiment. At the end of the treatments, the medium was discarded from plates and the cells were subsequently washed twice with phosphate buffered saline (PBS, Sigma). Cells were then incubated with CSFM containing water-soluble yellow 0.5 mg/ml of mitochondrial dye MTT for 3 hours at 37 °C in an atmosphere of 5 % CO₂. The solution was aspirated carefully and 100 µl of dimethylsulfoxid (DMSO) was added to dissolve the blue-colored formazan particles. Absorbance of samples from duplicate wells was measured by an ELISA reader (Sirio microplate reader, Seac Corp. Florence, Italy) at the wavelength of 570 nm representing the values in arbitrary unit (AU). Results are expressed as percentage of control values.

3.3.5. Annexin V and propidium iodide staining of cells

Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phospholipid phosphatidylserine to the external environment.

Annexin V is a Ca²⁺-dependent phospholipid binding protein that has a high affinity for phospholipid phosphatidylserine, and is useful for identifying apoptotic cells with exposed phospholipid phosphatidylserine. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas as the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing measurable apoptosis (72,73).

First, the medium was discarded and wells were washed twice with phosphate buffered saline (PBS, Sigma). Cells were removed from plates using a mixture of 0.25 % trypsin (Sigma, Hungary), 0.2 % ethylene-diamin tetra-acetate (EDTA; Serva, Hungary), 0.296 % sodium citrate, 0.6 % sodium chloride in distilled water. This medium was applied for 5 minutes at 37 °C. Removed cells were washed in cold PBS and were resuspended in binding buffer containing 10 mM Hepes NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂. Cell-count was determined in Bürker's chamber for achieving a dilution in which 1 ml of solution contains 10⁶ cells. 100 µl of buffer (10⁵ cells) was transferred into 5 ml round bottom polystyrene tubes. Cells were incubated for 15 minutes with fluorescein isothiocyanate (FITC) conjugated annexin V molecules and propidium iodide (PI) according to instruction of manufacturers. After this period of incubation, 400 µl of Annexin-binding buffer (BD Biosciences, Pharmingen, USA) was added to the tubes as described by manufacturers. The samples were immediately measured by BD FACS Calibur flow cytometer (BD Biosciences, USA) and analysed with cellquest software. Cells in each category are expressed as percentage of the total number of stained cells counted.

3.3.6. Measurement of cell signaling pathways

Phospho-JNK, phospho-p38, phospho-ERK/p42-44 and phospho-GSK-3β was quantified using intracellular staining for flow cytometry. Following the variant treatments in different groups, cells were harvested by trypsin–EDTA according to the protocol described at annexin V-PI staining. Cells were pelleted by centrifugation (175 g, 5 min) and then fixed in 2% formaldehyde in PBS for 10 min at 37 °C. After 1 min of chilling, cell suspensions were centrifuged again followed by permeabilization applying 90% methanol (Sigma, Hungary) for 30 min at 4 °C. Each tube of cells was rinsed twice with 0.5% BSA (Invitrogen, USA) and finally appropriate amounts of cells (0.5–5×10⁶) were resuspended in 0.5% BSA. Cells were then incubated for 10 min at room temperature. Subsequent to blocking of cells in BSA, appropriate dilution of primary antibody was added to the solution and was incubated for 1 hour at room temperature. Antibodies against phospho-JNK, phospho-p38, phospho-ERK/p42-44 and phospho-GSK-3β were from Cell Signaling Technology Inc. (USA), and from R&D System (USA) and they were used at dilutions 1:100 (phospho-JNK), 1:25 (phospho-p38), 1:100 (phospho-ERK/p42-44), and 1:100 (phospho- GSK-3β). After centrifugation, supernatant was carefully

aspirated and cells were resuspended in 100 μ l 0.5% BSA containing FITC conjugated secondary antibody (Goat anti-rabbit IgG; R&D System, USA) at a dilution of 1:20, and were incubated for 30 min. Fluorescent staining of samples was quantified by flow cytometric measurement of 5,000 cells. To determine the non-specific marking of cells, secondary antibody was applied for 30 min without primary antibody following permeabilization. Our results were analyzed by Cellquest software (BD Biosciences, USA), measuring the appearance of phospho-JNK, phospho-p38, phospho-ERK/p42-44, and phospho-GSK-3 β in the cells as mean fluorescence intensity (MFI).

3.3.7. Statistical analysis

All data are presented as mean \pm standard error of the mean (S.E.M). Differences between groups were assessed with one-way ANOVA and Student's t test and were considered significant when P-value was less than 0.05.

3.4. RESULTS

3.4.1. MTT assay results

An MTT assay was performed to measure the absolute number of living cells in the groups. In the control group, the amount of living cells was increased to 100%. EA alone reduced the ratio of living cells to $43.41 \pm 11.15\%$, measured by MTT assay. Both I/R and H₂O₂ alone caused a marked reduction in the amount of living cells. The effect of cell death was significantly stronger on EA administration in groups treated with H₂O₂ or exposed to I/R. (**Figure 4.**)

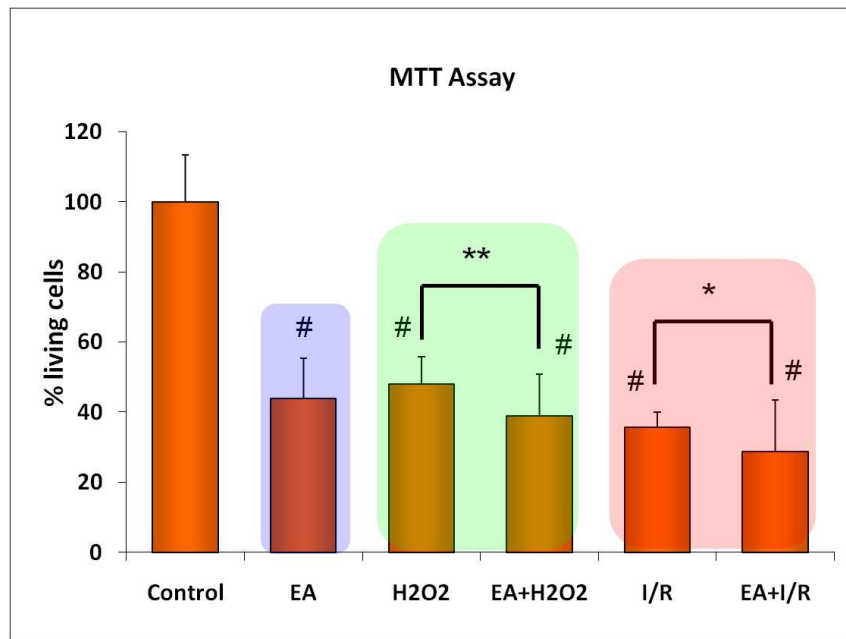


Figure 4. Viability of cardiomyocytes as measured by the MTT assay. # $p < 0,05$ compared with the control group. * $p < 0,05$; ** $p < 0,01$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion

3.4.2. Ratio of apoptosis

The control group had $85.7 \pm 1.94\%$ of intact, living cells (annexin V and PI negative) and $4.6 \pm 0.82\%$ of cells in the early phase of apoptosis (annexin V positive and PI negative). (**Figure 5.**) EA administration decreased the amount of living cells, and increased the percentage of apoptotic cells. A significant increase in the amount of apoptotic cells was observed in both the H_2O_2 -treated and I/R groups, with a lower number of living cells. (**Figures 5,6.**) When EA was added in groups treated with H_2O_2 or I/R, the quantity of apoptotic cells was further increased and the amount of living cells was decreased. Interestingly, EA increased the amount of necrotic cells (annexin V negative and PI positive) during I/R and decreased the number of living cells.

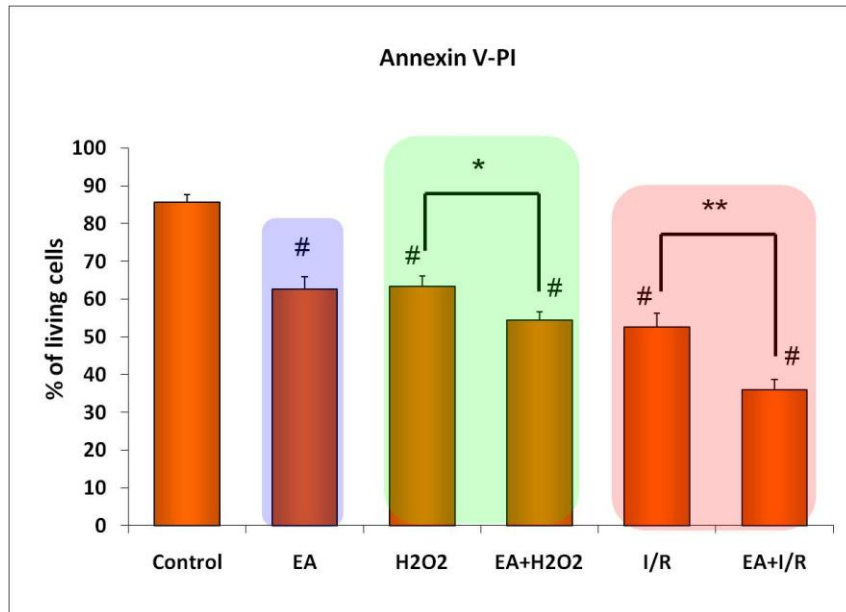


Figure 5. The mean percentage of living cells. Data expressed as mean percentage \pm SEM. # $p < 0,05$ compared with the control group. * $p < 0,05$; ** $p < 0,01$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion

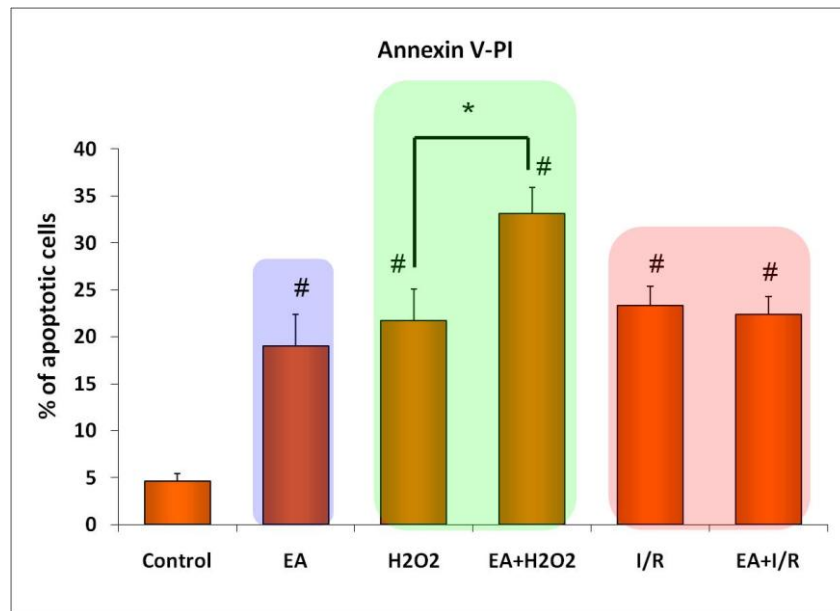


Figure 6. The mean percentage of apoptotic cells. Data are expressed as mean percentage \pm SEM. # $p < 0,05$ compared with the control group. * $p < 0,05$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion

3.4.3. Phosphorilation of mitogen activated protein kinases (MAPKs)

c-Jun N-terminal kinase (JNK) activation increased markedly on EA administration to cardiomyocytes. **(Figure 7.)** H₂O₂ treatment increased the level of activated JNK; however, this difference was not significant. I/R caused a noticeable increase in JNK activation. On the other hand, EA was capable of augmenting the activation of JNK significantly when cells were cotreated with H₂O₂ or when cells were exposed to I/R. **(Figure 7.)**

GST inhibition led to a significant increase in p38 activation related to nontreated cells. Both H₂O₂ incubation and I/R resulted in a significant increase in p38 MAP kinase activation. EA administration during I/R increased p38 activity to 357.57±5.39% of control values. Likewise, when cells were incubated with H₂O₂ together with EA, the level of phosphorylated p38 markedly increased; however, this difference was not statistically significant compared with the group treated with H₂O₂ alone. **(Figure 8.)**

Extracellular signal-regulated kinase (ERK) phosphorylation increased in GST-inhibited groups (incubated with EA) that were either treated with H₂O₂ or exposed to I/R, without any statistically significant difference. ANOVA failed to evaluate significant differences between groups. Moreover, the analysis of difference (using Student's *t* test) between the group receiving I/R and the group incubated with EA during I/R revealed statistically significant divergence. **(Figure 9.)**

Both administration of EA, H₂O₂ and I/R caused nonsignificant reduction of Akt activity. On the other hand, H₂O₂ treatment resulted in a more pronounced decrease (40.49±5.68%) of Akt phosphorylation when GST was inhibited by EA. **(Figure 10.)** There was no significant difference among groups as evaluated by ANOVA.

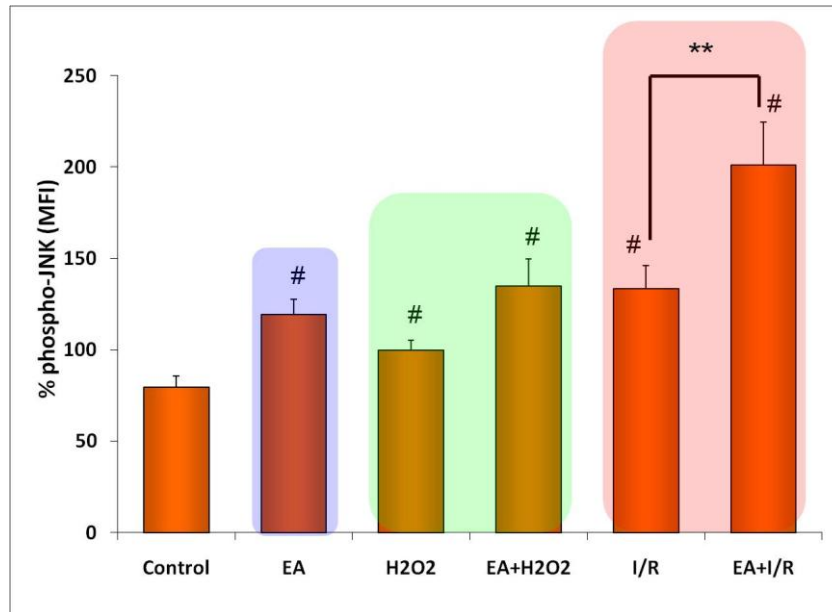


Figure 7. Phosphorylation of c-Jun N-terminal kinase (JNK) is demonstrated in cultured cardiomyocytes. # $p < 0,05$ compared with the control group. ** $p < 0,01$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion; MFI Mean fluorescence intensity

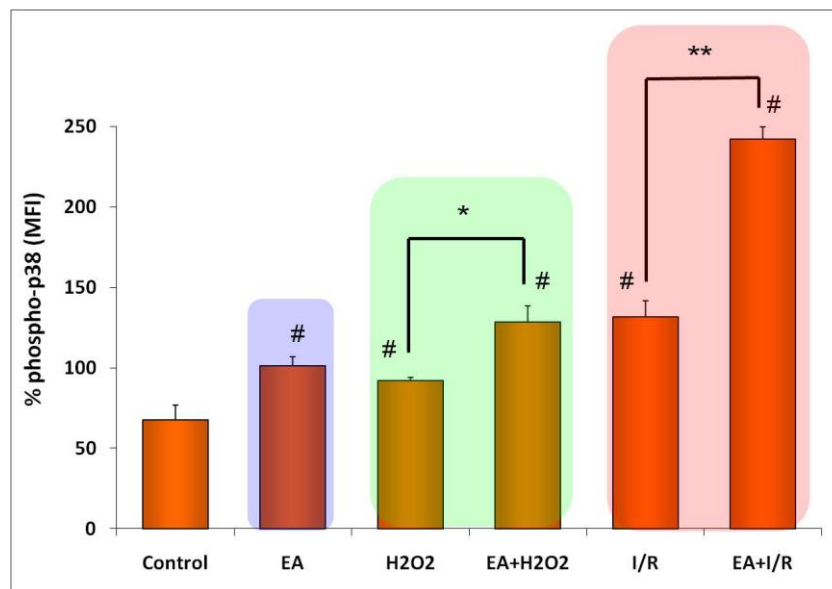


Figure 8. Phosphorylation of p38 (phospho-p38) mitogen-activated protein kinase is demonstrated in cultured cardiomyocytes. # $p < 0,05$ compared with the control group. * $p < 0,05$; ** $p < 0,01$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion; MFI Mean fluorescence intensity

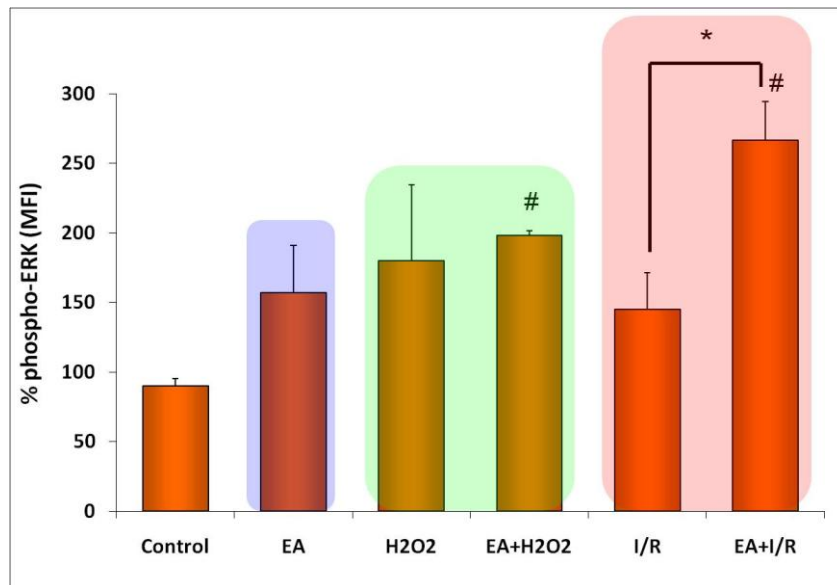


Figure 9. Phosphorylation of extracellular signal-regulated kinase (phospho-ERK) is demonstrated in cultured cardiomyocytes. # $p < 0,05$ compared with the control group. * $p < 0,05$ compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia an reperfusion; MFI Mean fluorescence intensity

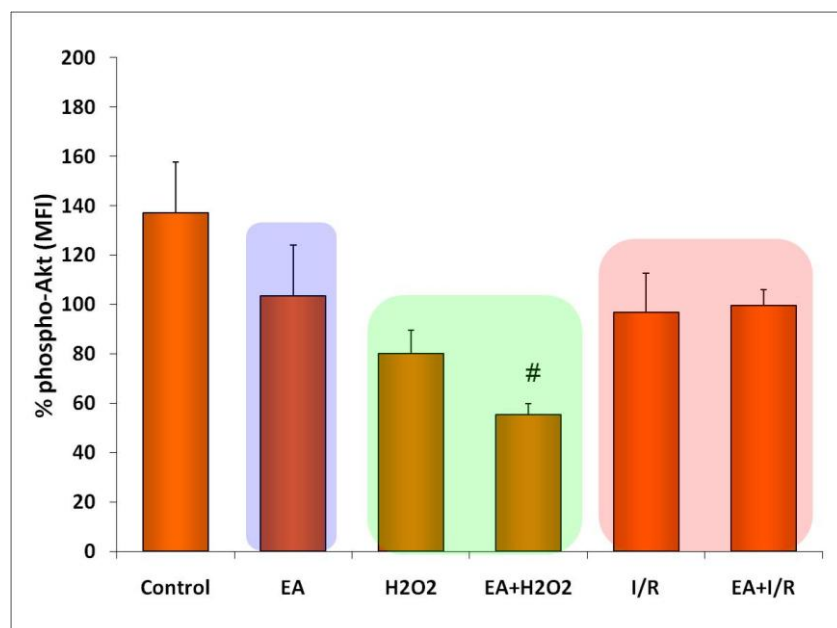


Figure 10. Phosphorylation of Akt (phospho-Akt) is demonstrated in cultured cardiomyocytes. # $p < 0,05$ compared with the control group. EA Ethacrynic acid; I/R Ischemia and reperfusion; MFI Mean fluorescence intensity

3.5. CONCLUSION

Pharmacological inhibition of GST could markedly exaggerate oxidative stress-induced apoptosis in cardiomyocytes. GST inhibition was associated with increased activation of MAP kinases under stress conditions.

A key determinant of the cellular response to oxidative stress relates to the level and molecular form of glutathione (74). A crucial factor that affects the level of glutathione is its utilization via GST (8). GSTs function by conjugating reduced GSH and catalyzing the attack on foreign compound or oxidative stress products, generally forming less-reactive materials that can be readily excreted.

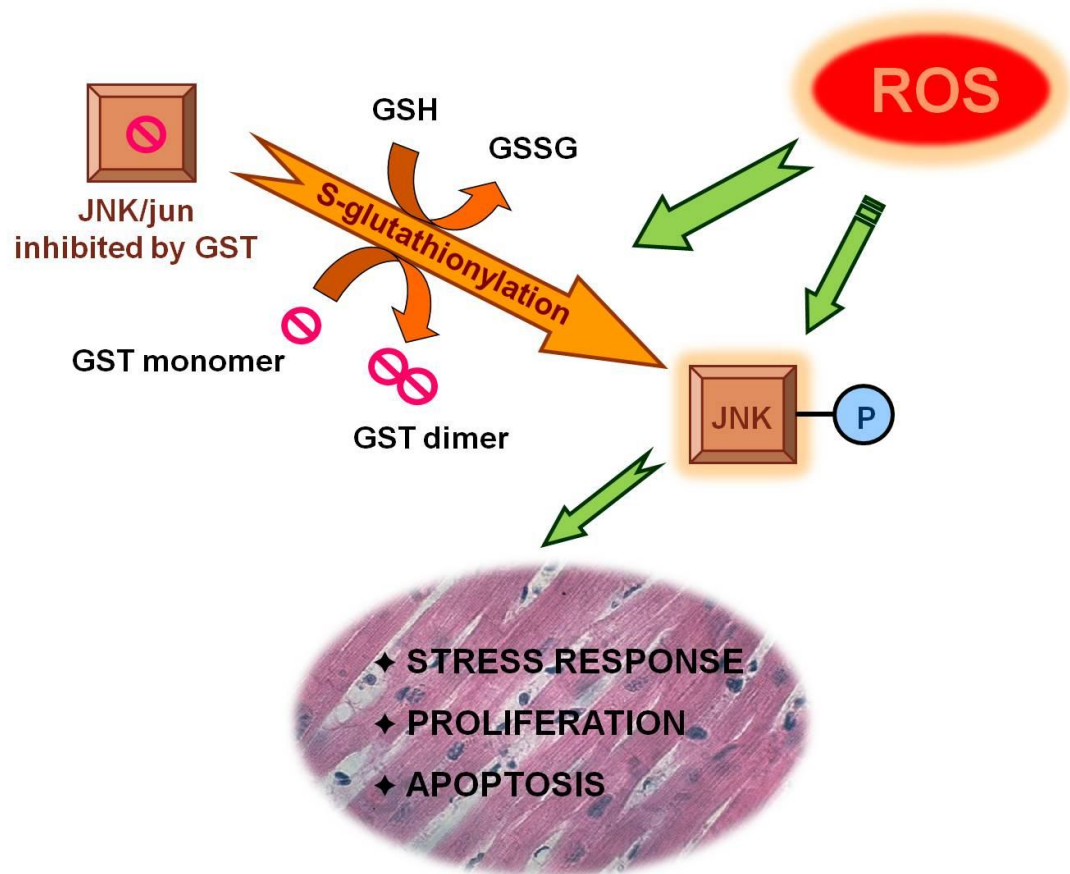
In our experiments administration of EA resulted in a marked increase of apoptotic cells, principally when cells were cotreated with H₂O₂. The amounts of necrotic cells were elevated following EA treatment and in the group receiving I/R and EA simultaneously. The increased level of reactive oxygen species and a more unfavourable glutathione state may exaggerate the intensity of insult and may explain the increased amount of necrotic cells in GST-inhibited groups during I/R.

On the other hand, GSTs associate with members of the mitogen activated protein kinase (MAPK) pathways involved in cell survival and death signaling (75). GST π was among the first isoenzymes found to inhibit c-Jun N-terminal kinase (JNK) through direct protein–protein interaction thus influencing cellular stress response and apoptosis (74). We have found that pharmacological inhibition of GST augments JNK activity itself. This could be explained by elimination of JNK sequestration within a protein complex with GST, and inhibition of S-glutathionylation. **(Figure 11.)**

The signalling pathway through p38 MAPK is activated by oxidative stress and is associated with cellular damage, mediation stress response and cytokine production. We found that oxidative injury and I/R cause noticeable induction of p38 activity in cardiomyocytes, which is further increased by EA administration. Our results regarding p38 activation on GST inhibition can be explained by the above-described processes.

According to our results, ERK is activated on GST inhibition in the presence of H₂O₂ administration or during reperfusion. The level of phosphorylated ERK of GST-inhibited cells receiving I/R exceeded the ERK phosphorylation level of cells that have undergone I/R alone. These findings may represent the association between ERK and GST.

Although similar relationships between the synthesis of GST and Akt have been well investigated, the effect of GST inhibition on Akt-mediated cellular survival has not been fully described (76,77). Our results failed to show any further association between GST inhibition and Akt activity. The hindered antioxidant, antitoxic defense of cells treated with EA may explicate the results described by us.



Townsend et al., Biomedicine & Pharmacotherapy 57 (2003) 145-155.

Figure 11. In unstressed cells, low JNK activity is maintained through a protein complex including pi GST, JNK, and c-Jun. Under conditions of oxidative stress, however pi GST·JNK complex dissociates, so that JNK is free to act on downstream gene targets.

4. INFLUENCE OF SELECTIVE MAPK AND GST INHIBITORS ON THE VIABILITY AND APOPTOSIS OF CARDIOMYOCYTES FOLLOWING OXIDATIVE STRESS

4.1. INTRODUCTION

It has been well investigated that oxidative stress is a major apoptotic stimulus in many cardiac diseases (50,51,52).

Among numerous defence mechanism against oxidative stress and ischaemic/reperfusion injury, the endogenous antioxidant enzyme glutathione S-transferase (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 (53,54).

GSTs play an important role as antioxidant enzyme modulating mitogen activated protein kinase (MAPK) pathways. The GSTs function to sequestering the kinase in a complex, serve a regulatory role, preventing cytotoxic ligands from interacting with their targets. Many studies have suggested that the mitogen activated protein kinases (MAPKs) may be important regulators of apoptosis in response to myocardial ischaemia/reperfusion.

Three major MAPKs, namely c-Jun NH₂-terminal protein kinase (JNK), p38 and extracellular signal-regulated protein kinase (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 (78,79). The pathways regulated by p38 and JNK contribute importantly to apoptosis. The mechanisms by which p38 and JNK induce apoptosis are largely cell and stimulus specific. ERK/p42-44 activation are protective against apoptotic cell death. (80,81,82,83,84,85).

Our pilot study has been conducted to examine the biological role of GST in cardiac myocytes under oxidative stress conditions. We found that that pharmacological inhibition of GST by EA augments the apoptosis as a result of oxidative stress and simulated ischaemic-reperfusion (SI/R) injury. The study showed that GST inhibition was associated with increased activation of MAP kinases under stress condition.

4.2. AIMS

The main objective of this study was to verify our previous results and identify the effect of MAPK (JNK, p38 and ERK) inhibitors regarding GST inhibition (with administration of ethacrynic acid) on the viability and apoptosis of cardiomyocytes when cells are exposed to various stress components of ischaemia and reperfusion (I/R).

4.3. MATERIALS AND METHODS

4.3.1. Cell culture from isolated neonatal cardiomyocytes

Primary culture of neonatal rat cardiomyocytes was prepared as described previously in part 3. Briefly, cells were obtained from ventricular myocytes of 2-4 day-old Wistar rats (Charles-River ltd., Hungary), using collagenase (Gibco™ Collagenase Type II, Invitrogen Corp., Carlsbad, CA, USA). Isolated cells were plated on collagen I-coated 24-well plates (BD Falcon) at the density of 2×10^5 cells/ml. Cells were incubated in DMEM/F12 medium (Sigma–Aldrich, USA) supplemented with 10 % of fetal bovine serum (Gibco, USA). The following day, when the cells attached to the plate firmly, the medium was replaced with complete serum free medium (CSFM).

4.3.2. MAPK inhibitors

JNK inhibitor:	SP600125	Sigma-Aldrich (S5567)
p38 inhibitor:	SB239063	Sigma-Aldrich (S0569)
ERK/p42-44 inhibitor:	U0126	Sigma-Aldrich (U120)

4.3.3. Experimental protocol

Primary culture of neonatal rat cardiomyocytes was prepared and divided in six experimental groups according to different exposers: control group of cells incubated in CSFM without treatment. In Group II, cells incubated in medium containing 150 μ M ethacrynic acid alone. In Group III, cells treated with 1mM H₂O₂ to simulate the oxidative stress. In group IV, cells exposed to 1mM H₂O₂ together with 150 μ M ethacrynic acid. In Group V, cells exposed to swapping ischemic buffer to CSFM to simulate ischaemic-

reperfusion injury. In Group VI, cells were treated to both sI/R and ethacrynic acid (EA). To antagonize the effect of JNK, p38 and ERK/p42-44 MAPKs, 10 μ M JNK inhibitor (SP600125), 10 μ M p38 inhibitor (SB239063) and 1 μ M ERK inhibitor (U0126) was added simultaneously in every group. (Table 1.)

Table 1. Experimental groups. Cells treated with 150 μ M EA for GST inhibition. Cells treated with 1mM H₂O₂ to simulate the oxidative stress. In groups receiving simulated I/R, cells were exposed to 1,5 hours of ischemia using ischaemic buffer followed by 2,5 hours of reperfusion using normal CSFM. Cells were exposed to mentioned concentration of chemicals for 4 hours. EA – Ethacrynic acid; H₂O₂ – Hydrogen-peroxide; I/R – Ischaemia and reperfusion.

Experimental groups	Without treatment	JNK inhibitor	p38 inhibitor	ERK/p42-44 inhibitor
I. Control (Cells were incubated in CSFM without treatment)	+			
		+		
			+	
				+
II. EA (Cells treated with 150 μ M ethacrynic acid)	+			
		+		
			+	
				+
III. H2O2 (Cells treated with 1 mM H ₂ O ₂)	+			
		+		
			+	
				+
IV. H2O2+EA (Oxidative stress + GST inhibition – double stress)	+			
		+		
			+	
				+
V. I/R (Cells exposed to 1,5 h ischaemia followed by 2,5 h reperfusion)	+			
		+		
			+	
				+
VI. I/R+EA (Simulated I/R + GST inhibition – double stress)	+			
		+		
			+	
				+

4.3.4. Determine the ratio of apoptosis

Ratio of apoptosis was evaluated after double staining with fluorescein isothiocyanate (FITC)-labeled annexin V (BD Biosciences, Pharmingen, USA) and propidium iodide (BD Biosciences, Pharmingen, USA) using flow cytometry, as described previously (73).

4.3.5. Statistical analysis

Up to four different cardiac myocyte preparations were studied. Data of the experiments are expressed as means \pm SE. Differences between the means were compared using two sample Student's t-test for significance. $p < 0,05$ was considered to be statistically significant.

4.4. RESULTS

4.4.1. MTT assay results

Viability of cardiomyocytes was determined by colorimetric MTT assay. In the control group without treatment, the amount of living cells was increased to 100%. JNK, p38 and ERK/p42-44 did not cause any significant changes in control groups without any stress effect. Ethacrynic acid, H₂O₂ and sI/R alone significantly reduced the ratio of living cells compared to control groups and only the p38 MAPK inhibition could significantly increase the viability of cells in H₂O₂-treated group. EA administration significantly enhanced the reduction of the viability of cells treated with H₂O₂ or exposed to sI/R compared to non treated cells and MAPK inhibitors could not increase significantly the percentage of living cells. **(Figure 12.)**

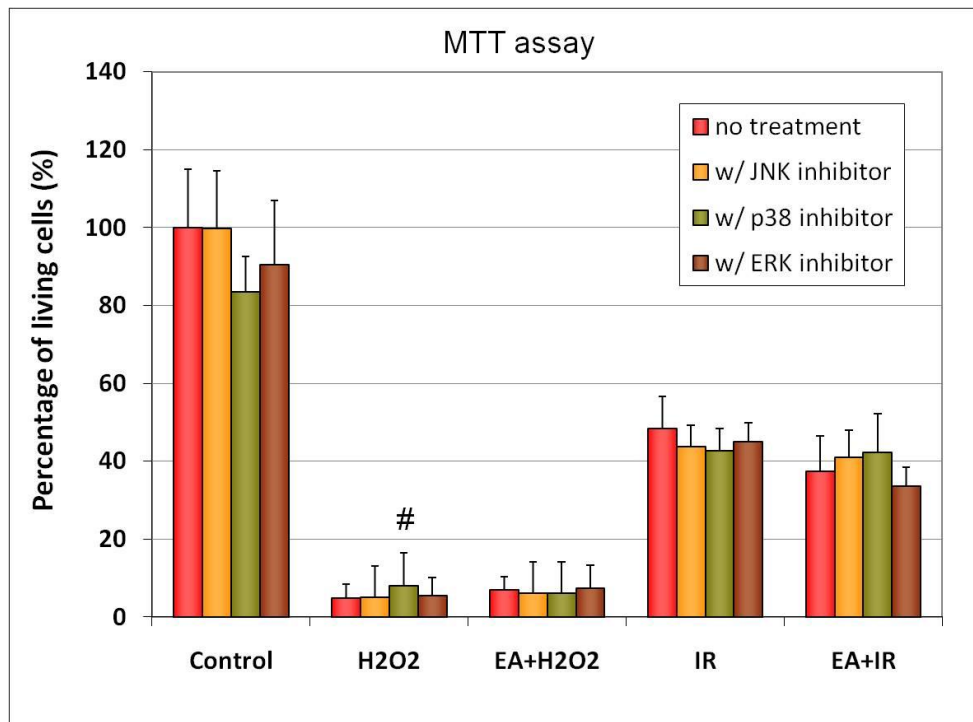


Figure 12. Viability of cardiomyocytes as measured by MTT assay. $p^{\#} < 0,05$ compared to the first column in every group without MAPK inhibitor. EA – Ethacrynic acid; H_2O_2 – Hydrogen-peroxide; I/R – Ischaemia and reperfusion.

4.4.2. Ratio of apoptosis

The non-treated control group had $92,4 \pm 1,47\%$ of intact, living cells (annexin V and PI negative) and $4.23 \pm 1,25\%$ of cells in the early phase of apoptosis (annexin V positive and PI negative). (**Figures 13,14.**) Control groups treated with different MAPK inhibitors had no significant differences compared to non-treated control group. EA, H_2O_2 administration and simulated I/R significantly decreased the amount of living cells ($p_{EA} = 0,0001$; $p_{H_2O_2} = 5,72E-09$; $p_{sI/R} = 2,65E-09$) and increased the percentage of apoptotic cells ($p_{EA} = 7,78E-05$; $p_{H_2O_2} = 1,68E-08$; $p_{sI/R} = 1,12E-08$) in non-treated control group. The inhibition of proapoptotic JNK and p38 MAPKs significantly ameliorates the cell viability and attenuates the rate of apoptosis in EA- and H_2O_2 -treated groups. On the other hand in these groups the inhibition of antiapoptotic ERK/p42-44 MAPK was significantly decreased the percentage of living cells ($p_{EA} = 0,035$; $p_{H_2O_2} = 2,92E-05$) and increased the amount of apoptotic cells ($p_{EA} = 0,05$; $p_{H_2O_2} = 1,36E-05$). In group, exposed to sI/R, only the p38 MAPK inhibitor was able to increase the percentage of viable cells ($p_{sI/R} = 0,022$) and diminish the rate of apoptosis ($p_{sI/R} = 0,018$) significantly. A significant increase in

amount of apoptotic cells was observed in both groups, exposed to double stress, H₂O₂+EA or sI/R+EA, with a lower number of living cells compared to non-treated group or groups exposed to H₂O₂ or sI/R. (**Figures 13,14.**) The administration of JNK or p38 MAPK inhibitors elevated the level of living cells and reduced the rate of apoptosis but not significantly in groups treated with H₂O₂ or exposed to sI/R supplemented with EA. When the antiapoptotic ERK/p42-44 MAPK was inhibited in groups treated with double stress, the amount of living cells was further decreased and the quantity of apoptotic cells was further increased.

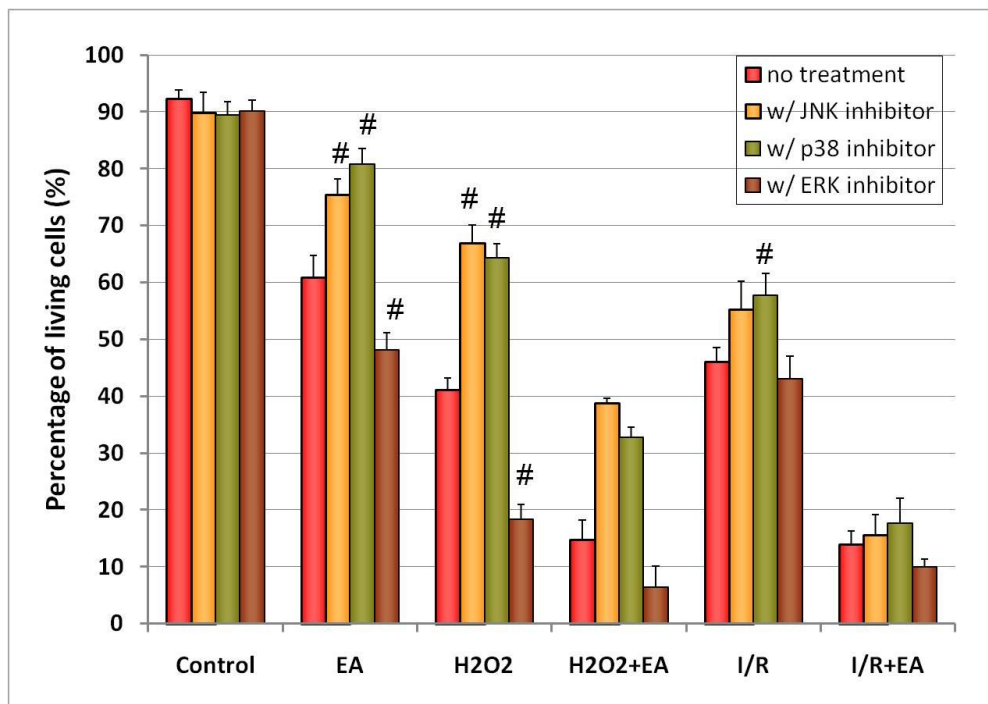


Figure 13. The mean percentage of living cells measured by flow cytometric assay. Data expressed as mean percentage \pm SEM. $p^{\#} < 0,05$ compared to the first column in every group without MAPK inhibitor. EA – Ethacrynic acid; H₂O₂ – Hydrogen-peroxide; I/R – Ischaemia and reperfusion.

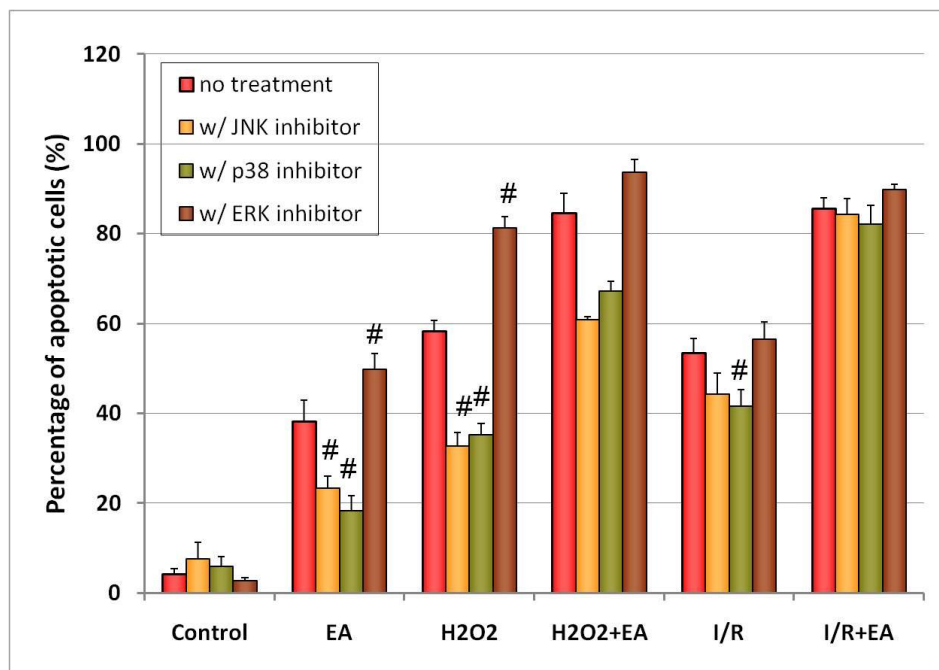


Figure 14. The mean percentage of apoptotic cells measured by flow cytometric assay. Data expressed as mean percentage \pm SEM. $p^{\#} < 0,05$ compared to the first column in every group without MAPK inhibitor. EA – Ethacrynic acid; H₂O₂ – Hydrogen-peroxide; I/R – Ischaemia and reperfusion.

4.5. CONCLUSION

The present study showed that pharmacological inhibition of proapoptotic JNK and p38 could significantly decrease the oxidative stress-induced apoptosis while the inhibition of antiapoptotic ERK/p42-44 markedly reduces the cell viability in cardiomyocytes. This effect of these MAPK inhibitors could not be observed in case of double stress (oxidative stress + GST inhibition).

To evaluate the significance of JNK, p38 and ERK/p42-44 and the possible link of GST to these MAPKs during oxidative stress, three specific inhibitors (SP600125, SB239063 and U0126) were used respectively. The SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK. SB239063 is a novel p38 inhibitor that exhibits improved kinase selectivity and *in vivo* activity compared with the other p38 inhibitors (86). To influence on ERK/p42-44 activity, we used U0126, which is a potent inhibitor of MEK1/2, an upstream regulator of the phosphorylation of ERK/p42-44 (87). According to

Olli Tenhunen et al. administration of 1 μ M U0126 significantly reduced the level of phospho-ERK. On the other hand, treatment with 1 μ M U0126 had no effect on the levels of phosphorylated p38 kinase and, similarly, the administration of SB239063 did not affect the level of phospho-ERK/p42-44 (88).

Oxidative stress-induced apoptotic cell death during reoxygenation in cultured cardiomyocytes and *in vivo* hearts during reperfusion has been linked to an increased expression in JNK and p38 MAPKs (81,82,83,84,85). We have found that pharmacological inhibition of the proapoptotic JNK and p38 MAPK significantly increase the cell viability and decrease the ratio of apoptosis in groups receiving GST inhibition or oxidative stress compared to groups without JNK and p38 inhibitors. On the other hand in groups treated with double stress (GST inhibition together with oxidative stress or I/R injury) this protective effect of JNK and p38 inhibitor was lost. These findings may represent the association between JNK, p38 MAPK and GST (89).

In many cell types the ERK/p42-44 cascade appears to mediate specifically cell growth and survival signals. According to our results, the antiapoptotic ERK/p42-44 inhibitor treatment significantly decrease the cell viability and increase the ratio of apoptosis in groups receiving GST inhibition or oxidative stress compared to groups for lack of ERK/p42-44 inhibitor. In our previous study we demonstrated that antiapoptotic ERK/p42-44 is activated on GST inhibition, in the presence of H₂O₂ administration or during reperfusion (90), But in our present study we could not observed elevation in the percentage of living cells following oxidative stress or GST inhibition, likely because of the ERK/p42-44 inhibitor treatment. But among ERK/p42-44 inhibited groups exposed to GST inhibition, oxidative stress and simulated I/R injury, the ratio of living cells was the highest in case of GST inhibition. These findings may represent the association between ERK/p42-44 and GST. On the other hand in our preceding study we also demonstrated that the level of phosphorylated ERK/p42-44 of GST-inhibited cells receiving sI/R exceeded the ERK/p42-44 phosphorylation level of cells that have undergone sI/R alone (90). Accordingly in this present examinations presumably because of ERK/p42-44 inhibition, in groups treated with double stress (GST inhibition together with oxidative stress or simulated I/R injury) the cardiomyocyte cell viability was diminished and the ratio of apoptosis was elevated but not significantly compared to groups in absence of ERK/p42-44 inhibitor. However a significant increase in amount of apoptotic cells was

observed in both groups, exposed to double stress, H₂O₂+EA or sI/R+EA, with a lower number of living cells compared to groups exposed to H₂O₂ or sI/R alone (89).

5. THE ROLE OF GST IN MECHANISMS OF ISCHAEMIC POSTCONDITIONING

5.1. INTRODUCTION

Oxidative stress can lead to apoptotic, necrotic disorders in cells, after ischaemic/reperfusion injury in any organs. Oxygen free radicals are highly reactive molecules with an unpaired electron, and are associated widely with ischemic/reperfusion injury (1). In large quantities they overwhelm the endogenous antioxidant systems or, if the antioxidant system is insufficient or damaged, they accelerate the oxidative stress (2). Among numerous defence mechanism against oxidative stress and ischaemic/reperfusion injury, glutathione and glutathione-S transferase (GST) are crucially involved in cellular response to stress, apoptosis and proliferation.

Glutathione transferases (GSTs) play a major role in protecting cells against damage from reactive oxygen species by conjugating them with glutathione so that they can be rapidly eliminated (91). Several GST isoenzymes are involved directly in controlling cellular mitogenic pathways that influence cell proliferation and apoptotic cell death (55,56,92,93,94).

A promising approach to cardioprotection termed “ischaemic postconditioning” (IPoC) has been described by Vinten-Johansen et al. (95) It contains short series of repetitive cycles of brief reperfusion and reocclusion of the coronary artery applied immediately at the onset of reperfusion, can be effective against reperfusion injury. They showed that the mechanisms involved in postconditioning protection take place within the first minutes of reperfusion (96).

5.2. AIMS

Postconditioning is controlled reperfusion (45), defined as a series of brief interruptions of ischemia/hypoxia applied at the very onset of reperfusion can protect the myocardium from ischaemic/reperfusion injury. The antioxidant glutathione S-transferase is crucially involved against oxidative stress and ischaemic/reperfusion injury. So we were concerned whether the pharmacologic inhibition of GST can influence the function and benefit of ischaemic postconditioning (IPoC) in vitro ischaemic/reperfusion injury. We

examined the efficiency of ischaemic postconditioning by assessing the cell viability and apoptosis in rat cardiomyocyte cell culture and the activation of mitogen activated protein (MAP) kinase pathways.

5.3. MATERIALS AND METHODS

5.3.1. Cell culture model

Primary culture of 2-4 day-old neonatal Wistar rat cardiomyocytes was prepared and transferred onto 24 and 96-well plates (BD Falcon) at the density of 200 000 cells/ml as described previously in part 3. In each well 2 ml cell solution was seeded. After 24 hours from plating, cell media was substituted with CSFM.

5.3.2. Experimental protocol

In our experiments neonatal cardiac cell cultures were listed in 6 groups: Group I, control group of cells, incubated in CSFM without treatment; Group II, cells exposed to simulated ischemia-reperfusion (I/R); Group III, cells treated with simulated ischemia-reperfusion together with ischaemic postconditioning (IPoC); Group IV, cell were treated with 150 μ M ethacrynic acid (EA) alone; Group V, cells exposed to simulated ischemia-reperfusion with 150 μ M ethacrynic acid; Group VI, cells exposed to simulated ischemia-reperfusion with ischaemic postconditioning and 150 μ M ethacrynic acid.

5.3.3. Ischemic postconditioning of the myocardium

Primary cardiomyocytes were utilized in a well established cellular model of ischemic postconditioning. Briefly, cells were exposed to simulated ischemic (SI) buffer for 1,5 hours followed by 5 minutes reperfusion and then another 5 minutes ischemic insult before the 2,5 hours reperfusion. While cardiomyocytes were under hypoxic conditions in SI buffer, control cells were incubated in complete serum free medium (CSFM) such as during reperfusion period. (**Figure 15.**)



Figure 15. Experimental protocol and effects of postconditioning with simulated ischemia/reperfusion and the glutathione S-transferase (GST) inhibitor EA treatments on cardiomyocytes. Cardiomyocytes were incubated in complete serum-free medium (CSFM) or with EA followed by 1,5 hours ischaemia and brief period of ischemia before 2,5 hours long reperfusion (R).

5.4. RESULTS

5.4.1. MTT assay results

MTT assay was performed to measure the absolute number of living cells in different groups. In a control group the amount of living cells was taken to 100 %. Both EA and simulated I/R alone caused marked reduction in amount of living cells. The effect of cell death was significantly stronger upon administration of EA in groups exposed to simulated I/R ($15,97 \pm 2,64$). Ischaemic postconditioning could significantly increase the percentage of living cells in case of simulated I/R ($p=0,023$), but this change was not observed when it is combined with EA treatment ($p=0,0015$). (**Figure 16.**)

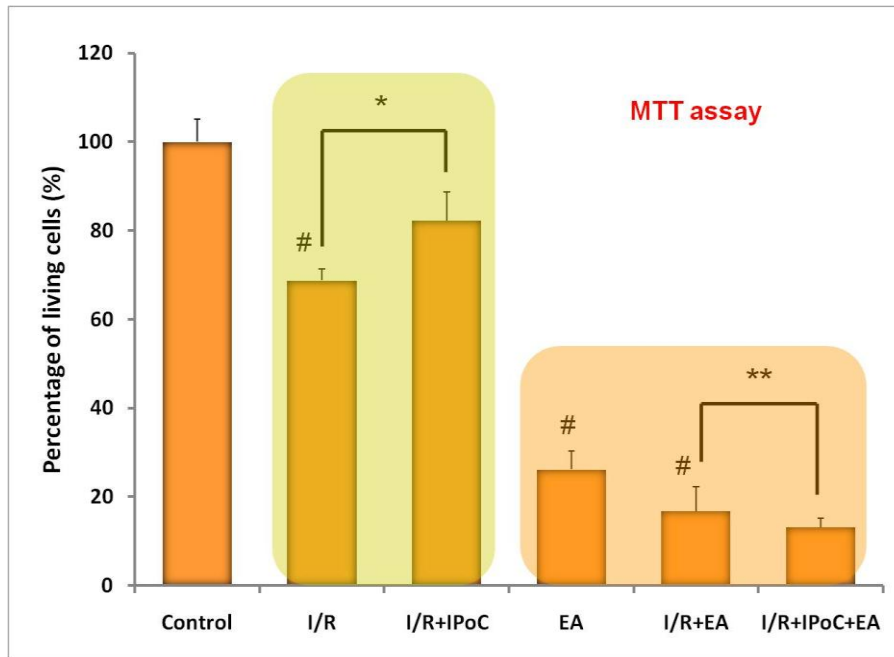


Figure 16. Viability of cardiomyocytes as measured by MTT assay. $p^{\#} < 0,05$ compared with the control group. $p^* < 0,05$ and $p^{**} < 0,01$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning.

5.4.2. Ratio of apoptosis

The control group had 83.54 ± 2.31 % of intact, living (annexin V and PI negative) cells and 7.8 ± 2.05 % of cells in early phase of apoptosis (annexin V positive and PI negative) (**Figure 17,18**). A significant increase of apoptotic cells was observed in both the EA-treated and simulated I/R groups with a lower number of living cells. When EA was added in simulated I/R groups the quantity of apoptotic cells was further increased with reduced amount of living cells (**Figure 17,18**). Ischaemic postconditioning resulted significant increase in the percentage of living cells and a significant decrease in the ratio of apoptotic cells in simulated I/R group while we could not detected this protective effect in simulated I/R group when EA co-treatment was applied (**Figure 17,18**).

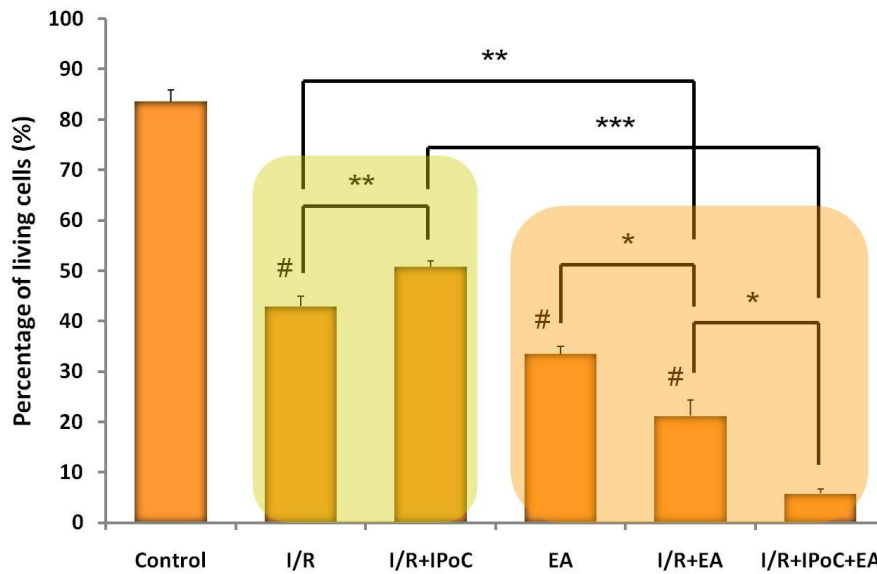


Figure 17. The mean percentage of living cells measured by flow cytometric assay. Data expressed as mean percentage \pm SEM. $p^{\#} < 0,05$ compared with the control group. $p^* < 0,05$ and $p^{**} < 0,01$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning.

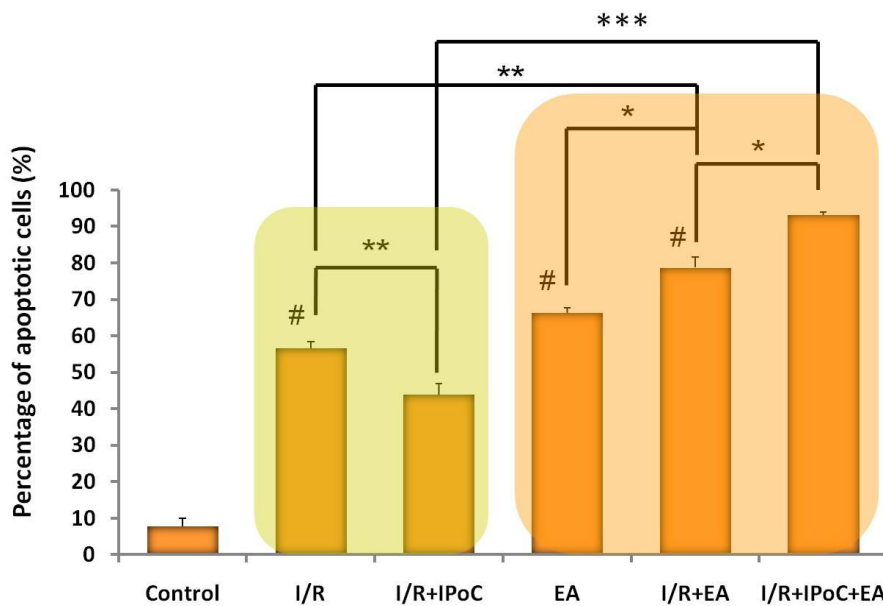


Figure 18. The mean percentage of apoptotic cells. Data expressed as mean percentage \pm SEM. $p^{\#} < 0,05$ compared with the control group. $p^* < 0,05$ and $p^{**} < 0,01$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning.

5.4.3. Phosphorylation of mitogen activated protein kinases (MAPKs)

JNK activation increased markedly upon administration of EA to cardiac myocytes and in simulated I/R group. **(Figure 19.)** EA administration resulted in further increase in JNK activation in cells exposed to simulated ischaemia and reperfusion. **(Figure 19.)** Ischaemic postconditioning decreased the JNK phosphorylation significantly in simulated I/R group while this change could not be detected in EA-treated simulated I/R group. **(Figure 19.)** Among the postconditioned groups the level of phosphorylated JNK was significantly higher when cardiomyocytes were treated with GST inhibitor EA.

Both, simulated I/R and GST inhibition led to significant increase in p38 activation related to non-treated cells. EA administration during simulated I/R resulted in further elevation in phosphorylation of p38. A significant decrease in p38 activation was detected in ischaemic postconditioned group compared to I/R group ($p=0,0037$). However, we could not observe similar, significant decrease between these groups if the cells treated with EA ($p=0,213$). Ischaemic postconditioning reduced significantly the level of phospho-p38 compared to same group receiving EA administration ($p=0,0072$). **(Figure 20.)**

ERK/p42-44 phosphorylation significantly increased in simulated I/R ($p=2,52E-10$) and GST inhibited ($p=1,14E-08$) groups. When cardiomyocytes were treated with both sI/R and EA we observed further elevation in the level of phosphorylated ERK/p42-44 compared to only GST inhibited group ($p=0,039$). The level of phosphorylated ERK/p42-44 of GST inhibited cells receiving simulated I/R was similar than the phospho-ERK/p42-44 level of cells undergone simulated I/R alone. Ischaemic postconditioning could significantly enhance the activation of ERK/p42-44 in simulated I/R group ($p=0,0012$) but we did not find significant change in case of double stress when simulated I/R combined with EA treatment using ischaemic postconditioning ($p=0,53$). **(Figure 21.)** The level of phospho-ERK/p42-44 was significantly higher in simple postconditioned group compared to the EA-treated group after postconditioning ($p=0,0009$).

The phosphorylation of GSK-3 β which causes the inhibition of this protein kinase itself and therefore prevents the opening of the mitochondrial permeability transition pore (mPTP), a mechanism preventing apoptotic cell death was significantly lower in simulated I/R group compared to control group ($p=0,0019$). Significantly increased GSK-3 β inactivation was observed in ischaemic postconditioned group compared to simulated I/R

group ($p=0,0393$). We measured significant decreasing in GSK-3 β inactivation in EA-treated group compared to control ($p=0,00047$) and simulated I/R ($p=0,0016$) groups. Further reduction in the level of phospho-GSK-3 β could be detected in groups treated with double stress (simulated I/R and GST inhibitor EA) compared to simulated I/R ($p=4,7E-07$) or EA treatment ($p=7,94E-05$) alone. We did not measure significantly higher phospho-GSK-3 β level in postconditioned group treated with double stress compared to I/R+EA group ($p=0,059$). GSK-3 β phosphorylation and thus the inactivation was significantly lower in case of EA administration ($p=0,0006$) compared the postconditioned groups to each other. (**Figure 22.**)

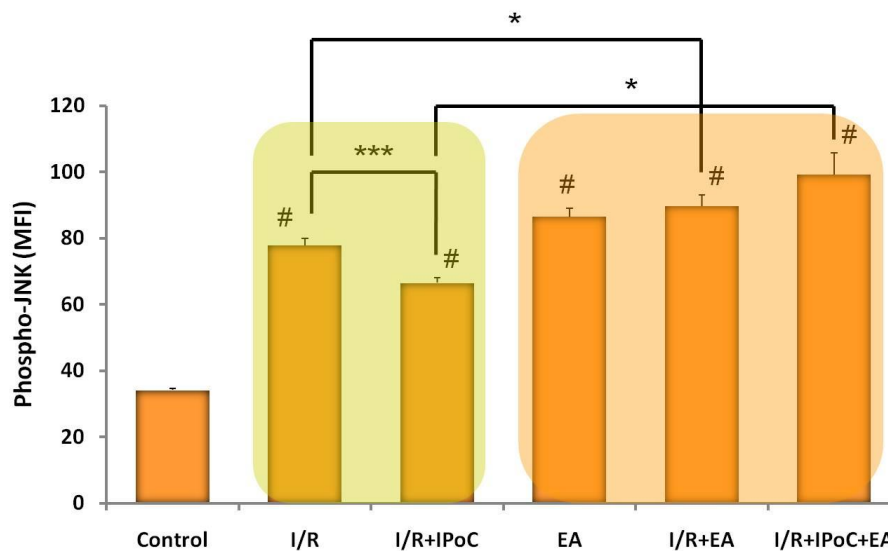


Figure 19. Phosphorylation of c-Jun N-terminal kinase (JNK) is demonstrated in cultured cardiomyocytes. $p^{\#}<0,05$ compared with the control group. $p^{***}<0,001$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning; MFI – Mean fluorescence intensity.

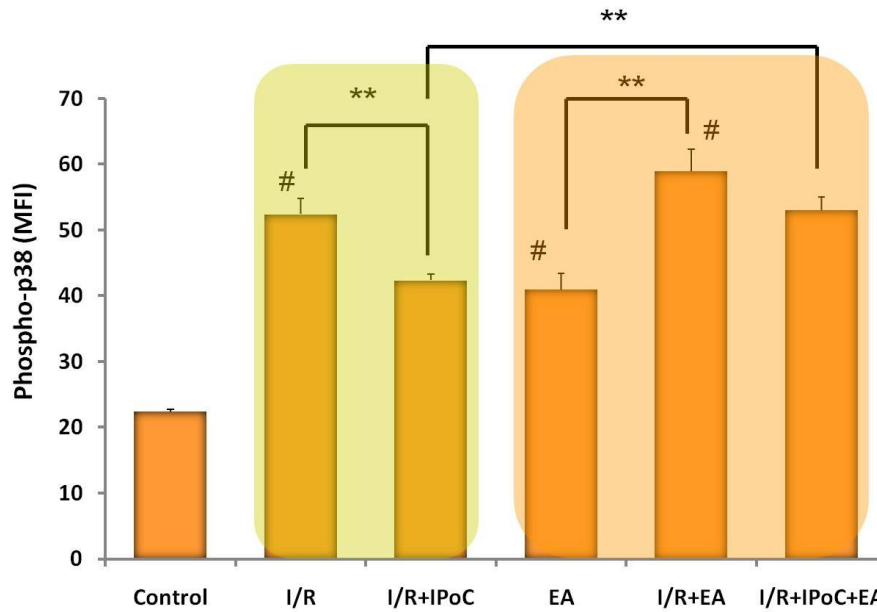


Figure 20. Phosphorylation of p38 (phospho-38) mitogen-activated protein kinase is demonstrated in cultured cardiomyocytes. $p^{\#} < 0,05$ compared with the control group. $p^* < 0,05$ and $p^{**} < 0,01$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning; MFI – Mean fluorescence intensity.

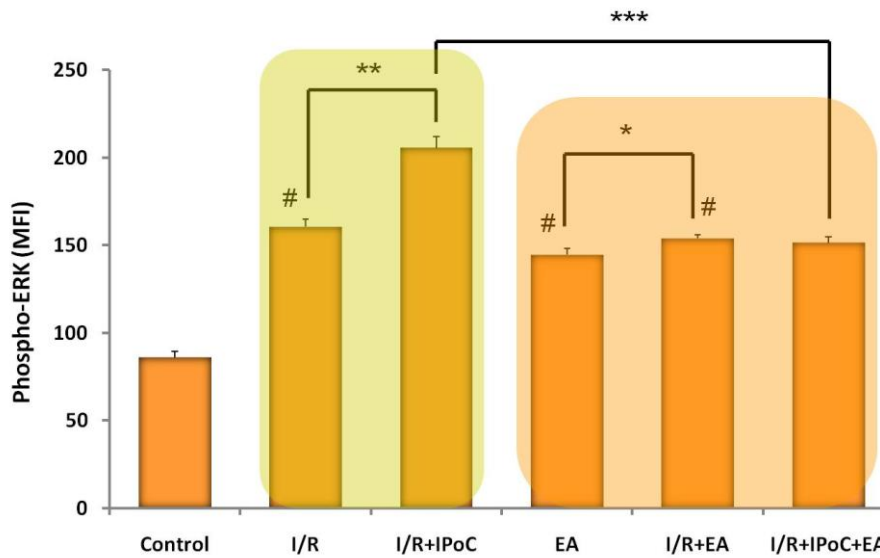


Figure 21. Phosphorylation of extracellular signal-regulated kinase (phospho-ERK/p42-44) is demonstrated in cultured cardiomyocytes. $p^{\#} < 0,05$ compared with the control group. $p^{**} < 0,01$ and $p^{***} < 0,001$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning; MFI – Mean fluorescence intensity.

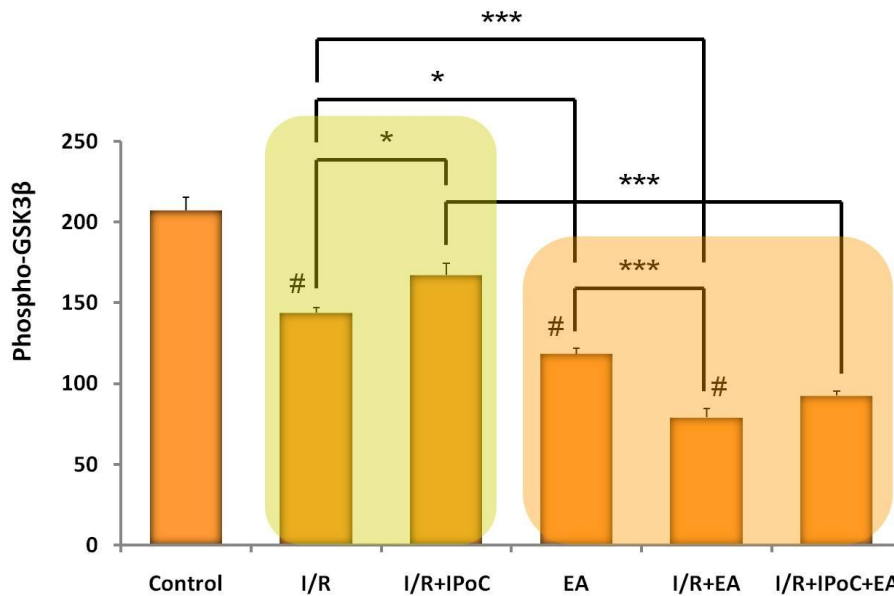


Figure 22. Phosphorylation of Glycogen synthase kinase-3 β (phospho-GSK-3 β) is demonstrated in cultured cardiomyocytes. $p^{\#} < 0,05$ compared with the control group. $p^{**} < 0,01$ and $p^{***} < 0,001$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning; MFI – Mean fluorescence intensity.

5.5. CONCLUSION

Present study showed that GST inhibition could markedly attenuate the protective effect of ischaemic postconditioning and resulted in increasing apoptosis in cardiomyocyte cell culture. GST inhibition was associated with different activation of MAP kinases and GSK3 β protein kinase in the process of ischaemic postconditioning.

In our experiment administration of GST inhibitor EA resulted in marked increase of apoptotic cells, principally when cells were co-treated with simulated I/R. While ischaemic postconditioning could decrease the ratio of apoptosis in simulated I/R group, this positive effect could not be detected in GST inhibited group receiving simulated I/R. The increased level of reactive oxygen species and more unfavourable glutathione state may aggravate the intensity of insult and may explain the increased amount of apoptotic cells in GST inhibited group during simulated I/R and IPoC (41). So far, the cardioprotection and the signal transduction pathways of postconditioning have been investigated mainly in healthy animal models and rarely in animals with clinical

pathologies. Iliodromitis et al. demonstrated that postconditioning is ineffective in limiting the infarct size in rabbits with hypercholesterolemia and an advanced stage of atherosclerosis (97). Ferdinandy et al. has demonstrated that comorbidities and aging accompanying coronary disease modify responses to ischemia/reperfusion and the cardioprotection conferred by preconditioning and postconditioning. They reviewed that aging or age-associated alterations (such as hypertrophy or remodeling) are associated with the loss or attenuation of cardioprotection by acute ischemic or pharmacological preconditioning as well as ischemic postconditioning (42).

GSTs have been shown to form protein:protein interactions with members of the mitogen activated protein (MAP) kinase pathway thereby serving a regulatory role in the balance between cell survival and apoptosis. By interacting directly with MAP kinases, including c-Jun N-terminal kinase 1 (JNK1), GSTs function to sequester the ligand in a complex, preventing interactions with their downstream targets (56,74,91,96). We have found that pharmacological inhibition of GST augments JNK activation by itself and abolish the protective effect of ischaemic postconditioning. This could be explained by elimination of the GST : JNK protein complex.

The MAPK p38 is a signaling protein that plays a critical role in coordinating cellular responses to stress, including oxidative stress that is characterized by the accumulation of increased levels of reactive oxygen species (ROS) within the cell. We found that simulated I/R treatment cause noticeable induction of p38 activation in cardiomyocytes, which was further increased by administration of EA. Consistent with the literature ischaemic postconditioning was able to decrease significantly the phosphorylation of p38 in sI/R group while we observed similar but not significant decrease in sI/R group treated with GST inhibitor EA, accordingly the relationship is supposed between GST and p38 which led to abolished effect of IPoC.

In many cell types the ERK/p42-44 cascade appears to mediate specifically cell growth and survival signals. For instance, it has been shown that inhibition of ERK/p42-44 enhances ischaemia-reperfusion induced apoptosis and that sustained activation of this kinase during simulated ischaemia mediates adaptive cytoprotection in cultured neonatal cardiomyocytes (98). According to our results ERK/p42-44 activation increased upon GST inhibition during reperfusion, like in 'Conclusion' of third chapter. This may presume the induction of this protective pathway due to oxidative stress. In our experiment the level of antiapoptotic phospho-ERK/p42-44 was significantly higher

following IPoC – consistent with previous studies – but this change was not shown in the presence of GST inhibitor ethacrynic acid. Although the activation of ERK/p42-44 increased due to GST inhibition or IPoC the elevation of this antiapoptotic MAPK was lost in case of double stress, probably cause of the extended stress.

Glycogen synthase kinase-3 β (GSK-3 β), a protein kinase linked to the regulation of a variety of cellular functions including glycogen metabolism, gene expression, and cellular survival, could either be considered as a specific downstream target of the RISK pathway or indeed as a component of the RISK pathway. We detected significant reduction in the level of phospho-GSK-3 β following simulated I/R treatment showing the harmful effect of I/R, but coincidentally with the literature the inactivation of GSK-3 β increased in case of IPoC. In case of double stress (sI/R+EA) we observed significant reduction in the phosphorylation of GSK-3 β , additionally IPoC was not able to significantly enhanced the inactivation of GSK-3 β . This result may presume the association between GSK-3 β and GST. The enhanced phosphorylation of GSK-3 β and ERK/p42-44, which is found in postconditioning under control conditions and is hypothesized to be involved in the signal transduction pathway of postconditioning, is lost in the metabolic syndrome (43). This data may represent the fact that the protective effect of ischaemic postconditioning is lost in case of double stress.

6. DISCUSSION

The tripeptide glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role in cellular defense against oxidative stress in cells. Under oxidative stress, GSH reacts either as an electron donor to neutralize hydrogen peroxides and lipoperoxides or as a direct oxygen free radical scavenger; this results in its depletion and excess of oxidized glutathione (GSSG). Therefore, the determination of the GSH/GSSG ratio and the quantification of GSSG are useful indicators of oxidative stress. Because of its important antioxidant properties, GSH is known to play a pivotal role in myocardial protection against ischemia/reperfusion (I/R) (99). GSTs catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds in order to inactivate them and facilitate their excretion from the body (100). In general, the reactions catalyzed by GSTs are considered detoxifying, and serve to protect cellular macromolecules from damage caused by oxidative stress and cytotoxic agents (101).

In the first series of our experiments we found that ethacrynic acid, I/R and H₂O₂ alone caused significant reduction in amount of living cells and significant increase in the ratio of apoptotic cells. GST inhibition by EA administration further decreased significantly the amount of living cells and further increased significantly the percentage of apoptotic cells in groups treated with H₂O₂ or exposed to I/R.

GSTs play a regulatory role in the mitogen-activated protein (MAP) kinase pathway that participates in cellular survival and death signals via protein : protein interactions. JNK is a proapoptotic MAP kinase mediates cytotoxicity in various conditions including I/R and oxidative, nitrosative stress, and involved in stress response, apoptosis, inflammation, and cellular differentiation and proliferation (44,45). The phosphorylation of JNK activates c-Jun, resulting in subsequent activation of downstream effectors. In unstressed cells, low JNK catalytic activity is maintained through its sequestration within a protein complex that includes pi GST, JNK, and c-Jun thus it is presumed that the dimeric form of GSTp is responsible for the regulatory control of JNK (43). Under conditions of oxidative or nitrosative stress, however [during which all three of these proteins are S-glutathionylated (44,46)], the pi GST·JNK complex dissociates, so that JNK regain its activity by phosphorylation and free to act on downstream gene targets, whereas the pi GST undergoes oligomerization (47,56). (Figure 11.) On the other hand in unstressed cells

effective inhibition of GST may cause oxidative injury due to hindered elimination of trivially developing oxidant and toxic materials. This may occur as a result of JNK phosphorylation. It has been already described that GST knockout mice have high basal JNK activity furthermore treatment of cells with potent GST inhibitor causes activation of JNK (53,102). In our experiments JNK activation increased markedly by EA exposure to cardiac myocytes and significantly augmented the activation of JNK both when cells were co-treated with H₂O₂ or when cells were exposed to I/R. It has already been described that GST knockout mice have high basal JNK activity; furthermore, treatment of cells with a potent GST inhibitor causes activation of JNK (53,102). Inhibition of GST led to significant increase in p38 activation versus non-treated cells. Both H₂O₂ incubation and simulated ischaemia-reperfusion resulted in significant increase of p38 MAP kinase activation. EA administration during sI/R or incubation together with H₂O₂ increased markedly the level of phosphorylated p38. ERK phosphorylation increased in GST inhibited groups (incubated with EA) either treated with H₂O₂ or exposed to I/R. It has already been described that an immortalized fibroblast isolated from a GST mu genotype expressed significantly elevated ERK activity. Moreover, treatment with a potent GST inhibitor resulted in activation of ERK (102), and vice versa, the transcriptional induction of the GST gene is orchestrated by signalling pathways, such as ERK, which might deteriorate due to GST inhibition (76). Active Akt/PKB is a serine/threonine kinase that, like ERK/p42-44, is important in response to growth factors and oxidative stress. H₂O₂ treatment resulted in pronounced decrease of Akt phosphorylation when GST was inhibited by EA.

In the second series of our experiments we found that pharmacological inhibition of proapoptotic JNK and p38 MAPKs diminish apoptosis while the inhibition of antiapoptotic ERK/p42-44 MAPK augments apoptosis as a result of GST inhibition even as oxidative injury and simulated I/R. Thus this apoptotic signaling is presumably mediated by JNK, p38 and ERK/p42-44 MAPKs pathways. The protective effect of the inhibition of proapoptotic JNK and p38 MAPKs was lost in groups, exposed to double stress (GST inhibition together with oxidative stress or simulated I/R injury), so GST activity is required for survival of cultured cardiomyocytes under stress conditions.

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation. The mammalian MAPK

family consists of c-Jun NH₂-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK/p42-44). Although there are conflicting reports on the role of MAPKs in death or survival after stress (80), it is commonly agreed that JNK and p38 MAPKs appear to be pro-apoptotic in many cell types while ERKs are the modulators of cell survival after reperfusion. (81,82,83,84,85).

In the third series of our experiments GST inhibition markedly attenuate the protective effect of IPoC which resulted in increasing apoptosis and decreasing cardiomyocyte cell viability. The proapoptotic JNK activation was significantly elevated and the proapoptotic p38 phosphorylation was not be able to significantly decrease in GST inhibited group despite of IPoC. Among same experimental conditions, ischaemic postconditioning could significantly enhance the antiapoptotic ERK/p42-44 activity in simulated I/R group but we did not find significant change in case of double stress when simulated I/R combined with EA treatment. The ratio of phospho-GSK-3 β was significantly higher in IPoC group compared to I/R group, but we could not observed similar significant elevation between these groups if the cells treated with EA.

Reperfusion injury to the myocardium results at least in part from oxygen free radicals (ROS) released from the ischemic tissue upon deoxygenation. This damage may be or may not be reversible, depending on the severity and duration of the ischemic period (103). Reversible damage is caused by combinations of free radical attack and transient calcium overload (104); irreversible injury occurs when the ischemic period is extended and severe; it is also mediated by oxidative stress and results in myocardial cell death through necrosis or apoptosis (105).

Ischaemic preconditioning (IPC) is a well known strategy to protect heart against ischaemia and reperfusion injury (34). However, unpredictability of clinical acute myocardial infarction precludes the application of preconditioning.

Recently, Zhao et al. (35) have reported that a short series of repetitive cycles of brief reperfusion and reocclusion of the coronary artery applied immediately at the onset of reperfusion, termed “postconditioning,” was as effective as preconditioning. They showed that the mechanisms involved in postconditioning protection take place within the first minutes of reperfusion (36). It may have greater clinical potential than preconditioning (37).

Evidence from in vitro and in vivo models has shown that death kinases such as the p38 and JNK MAPKs linked to myocardial injury after ischaemia and reperfusion are also

activated in response to stimuli present at reperfusion such as inflammatory cytokines and oxidants (80,85,92). There is little information on the modulation of death kinases in postconditioning. In a preliminary report by Zhao et al. (106) using isolated neonatal rat cardiomyocytes, intermittent reoxygenation and hypoxia (“hypoxic postconditioning”) inhibited the expression of p38 and JNK mitogen-activated protein kinases. According to Sun et al. attenuation in superoxide anion generation by postconditioning after hypoxia and reoxygenation, the expression and activation of JNK and p38 MAPKs are attenuated suggesting that modulation of MAPK signalling pathways are largely involved in postconditioning-induced protection (107).

According to Lazou et al. the cardioprotective effects of ischaemic preconditioning correlates with the activation of ERK/p42-44 during reperfusion (108). On the other hand ERK/p42-44 plays a pivotal role not only in preconditioning but also in postconditioning. In 1999, Yellon and co-authors (109) introduced the concept of a pro-survival reperfusion signalling pathway, which they subsequently termed the ‘Reperfusion Injury Salvage Kinase’ (RISK) pathway. The pharmacological activation of pro-survival kinases, such ERK/p42-44, at the immediate onset of myocardial reperfusion reduced infarct size by 40–50% (38,39). Successive studies have also confirmed the role for ERK/p42-44 in the setting of postconditioning in both non-diseased animal hearts, as well as in post-infarct remodelling (47,110). Interestingly, obese mice have been reported to be resistant to IPoC protection, and this finding was associated with insufficient activation of the RISK pathway in the hearts harvested from obese animals compared to control ones (111).

In postconditioning at least two signal transduction pathways, the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase pathways, are contributing to cardioprotection (112). These pathways converge, and PI3K causes the phosphorylation and thereby the inhibition of glycogen synthase kinase-3 β (GSK-3 β) (113). GSK-3 β -inhibition prevents the opening of the mitochondrial permeability transition pore (mPTP). Inhibition of mPTP opening at reperfusion, reduces infarct size due to inhibits the release of cytochrome C from mitochondria, a mechanism preventing apoptotic cell death so it may perhaps be the final effectors of cardioprotection (43,114,115). It has already shown that mice containing a mutant form of GSK-3 β , which cannot be phosphorylated and thereby inhibited, were resistant to the myocardial infarct-limiting effects of postconditioning, suggesting that GSK-3 β inactivation is required for postconditioning (49).

In sum, present study showed that inhibition of GST by EA augments the apoptosis as a result of simulated I/R furthermore abolish the protective effect of ischaemic postconditioning and this is presumably mediated by JNK, p38, ERK/p42-44 and GSK-3 β signaling pathways because the activities of these kinases change on this way during ischaemic postconditioning. GST activity is required for survival of cultured cardiomyocytes under stress conditions. These findings highlight the important role of GST in protection against oxidative stress likely not only in experimental conditions but in different pathological disorders in human beings thus serve as basis of (1) further studies investigating in vivo effect of GST inhibition, (2) clinical studies to investigate the role of GST on myocardial damage under different pathological conditions and (3) whether GST 0 genotype is associated with susceptibility of reperfusion injury and also abolished mechanisms of ischaemic postconditioning.

7. NOVEL FINDINGS

- 1) Firstly we demonstrated that the pharmacological inhibition of GST could markedly exaggerate oxidative stress-induced apoptosis in isolated cardiomyocytes, so GST activity is required for survival of cultured cardiomyocytes under stress conditions. Furthermore GST inhibition was associated with different activation of MAP kinases regulating these pathways under stress conditions.
- 2) We detected, using MAPK inhibitors, that GST plays crucial role among pro- and antiapoptotic MAPKs in the process of oxidative stress-induced apoptosis in cardiomyocyte cell culture.
- 3) We are the first who demonstrated that GST inhibition could markedly attenuate the protective effect of ischaemic postconditioning and resulted in increasing apoptosis in isolated cardiomyocytes. It was clarified that in the process of postconditioning GST inhibition is in the close association with activation of different MAP kinases and protein kinases.

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9. PUBLICATIONS AND PRESENTATIONS

9.1. Publications related to the thesis

1. L. Sínay, M. Kürthy, Sz. Horváth, E. Arató, M. Shafiei, J. Lantos, S. Ferencz, A. Bátor, **B. Balatonyi**, Zs. Verzár, B. Sütő, L. Kollár, Gy. Wéber, E. Róth, G. Jancsó. Ischaemic postconditioning reduces peroxide formation, cytokine expression and leukocyte activation in reperfusion injury after abdominal aortic surgery in rat model. *Clinical Hemorheology and Microcirculation*, 40 (2008) 133-142.

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2. 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. Róth, 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.

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3. E. Róth, 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.

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5. **Borbála Balatonyi**, Balázs Gasz, Viktória Kovács, János Lantos, Gábor Jancsó, Nándor Marczin, Erzsébet Róth. The role of the inhibition of Glutathione S-transferase /GST/ in the protective mechanisms of ischemic postconditioning.

IF.: 1,953

- 6. Borbála Balatonyi**, Balázs Gasz, Viktória Kovács, János Lantos, Gábor Jancsó, Nándor Marczin, Erzsébet Róth. Influence of mapk inhibitors on the oxidative stress of isolated cardiomyocytes.

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9.2. Publications not related to the thesis

1. Cserepes Barbara, Jancsó Gábor, Gasz Balázs, Rácz Boglárka, **Balatonyi Borbála**, Gaszner Balázs, Lantos János, Róth Erzsébet. Stressz-regulátor neuropeptid sejtprotektív hatása iszkémiás-reperfúziós károsodással szemben izolált szívmusclesejteken.

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2. Horváth Szabolcs, Gál István, Rákóczi István, Jávor Szaniszló, **Balatonyi Borbála**, Takács Ildikó, Ferencz Andrea, Ferencz Sándor, Wéber György. Transvaginalis cholecystectomy állatmodellen – kezdeti tapasztalataink.

Magyar Sebészet 2009; 62(3): 120-124.

3. Ferencz Sándor, Mangold Viktória, Dérczy Katalin, Takács Ildikó, **Balatonyi Borbála**, Horváth Szabolcs, Jávor Szaniszló, Rickard Branemark, Horváth Örs Péter, Róth Erzsébet, Wéber György. Alsóvégtag amputált érbetegek új protetizációs lehetősége: kezdeti tapasztalataink az összeintegrációs technikával.

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4. Racz B, Reglodi D, Horvath G, Szigeti A, **Balatonyi B**, Roth E, Weber G, Alotti N, Toth G, Gasz B. Protective Effect of PACAP Against Doxorubicin-Induced Cell Death in Cardiomyocyte Culture.

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6. Ildikó Takács, Szabolcs Horváth, Ágnes Molnár, Sarolta Gáspár, Rebeka Hajós, Ágnes Metzker, Péter Kóbor, János Lantos, Szaniszló Jávör, **Borbála Balatonyi**, György Szekeres, Erzsébet Róth, György Wéber. Comparative immunohistochemical study of tissue integration of macroporous and laminar surgical meshes
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7. Szaniszló Jávör, Koba Shanava, Enikő Hocsák, Mária Kürthy, János Lantos, Balázs Borsiczky, Ildikó Takács, Szabolcs Horváth, **Borbála Balatonyi**, Sándor Ferencz, Andrea Ferencz, Erzsébet Róth, György Wéber. Preconditioning is a method that may reduce the negative side effect of pneumoperitoneum
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9.3. Abstracts

1. Cserepes Barbara, Jancsó Gábor, Gasz Balázs, Rácz Boglárka, Ferencz Andrea, **Balatonyi Borbála**, Gaszner Balázs, Róth Erzsébet. Az urocortin a szívizomzat iszkémiás prekondicionálásában. (Poszter)
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2. Dr. Jancsó Gábor, Dr. Sínay László, Kürthy Mária, Dr. Lantos János, Bátor Anna, Németh Gábor, **Balatonyi Borbála**, Dr. Arató Endre, Prof. Dr. Róth Erzsébet.

Iszkémiás posztkondicionálás hatása hasi aorta okklúziót-reperfúziót követő oxidatív stressz mértékére.

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4. **Balatonyi Borbála**, Cserepes Barbara, Jancsó Gábor, Rácz Boglárka, Bertók Szabolcs, Ferencz Sándor, Horváth Szabolcs, Kürthy Mária, Lantos János, Róth Erzsébet. Az urocortin expresszió dinamikájának változása izolált szívizomsejteken iszkémiás prekondicionáló stimulust követően. (Poszter)
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