Investigations of pathological conditions and circulation during oncological reconstructive surgeries

PhD thesis

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Pécs, 2018
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**ABBREVIATIONS**

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<tr>
<td>Akt (PKB)</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>BFSP1</td>
<td>Beaded Filament Structural Protein 1</td>
</tr>
<tr>
<td>BSA-TBS</td>
<td>Bovine serum albumin-TRIS buffered saline</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleotic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Deltopectoral flap</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiograph</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>Elongation index</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERec</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HO-</td>
<td>Hydroxyl anion</td>
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I/R  Ischemia-reperfusion
IFs  Intermediate filaments
IL-1  Interleukine-1
IL-6  Interleukine-6
IL-8  Interleukin 8
iNOS  Inducible nitric oxide synthase
IPostC  Ischemic postconditioning
IPreC  Ischemic preconditioning
IRI  Ischemia-reperfusion injury
JNK  C-Jun N-terminal kinase
MAPK  Mitogen activated protein kinase
MCP-1  Monocyte chemoattractant protein 1
MDA  Malondialdehyde
MHC  Major histocompatibility complex
mPTP  Mitochondrial permeability transition pore
MS  Mass spectrometry
MSch  Medical School
NFkB  Nuclear factor kappa B
NK  Natural killer
NO  Nitric oxide
O2-  Superoxide anion
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PI3-kinase  Phosphatidylinositol 3 kinase
<table>
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<td>Protein kinase C</td>
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<tr>
<td>PKG</td>
<td>Protein kinase G</td>
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<tr>
<td>PMMC</td>
<td>Pectoral major myocutaneous flap</td>
</tr>
<tr>
<td>PMN cells</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td>PR</td>
<td>Progesteron receptor</td>
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<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
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<tr>
<td>RISK</td>
<td>Reperfusion injury salvage kinase</td>
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<tr>
<td>ROI</td>
<td>Reactive oxygen intermediers</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SH -</td>
<td>Thiol group</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>SS</td>
<td>Shear stress</td>
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<tr>
<td>TMZ</td>
<td>Trimetazidine</td>
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<tr>
<td>TNF-alpha</td>
<td>Tumor necrosis factor-alpha</td>
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<tr>
<td>TRAM</td>
<td>Transversus rectus abdominis muscle flap</td>
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<tr>
<td>TRIS</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
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<td>UP</td>
<td>University of Pécs, Hungary</td>
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1 INTRODUCTION

Cancer has a major impact on society across the world. In 2012, an estimated 14.1 million new cases of cancer occurred worldwide, of these 7.4 million cases were in men and 6.7 million in women. This number is expected to increase to 24 million by 2035. The global burden of cancer increases continuously and largely, because of the aging and growth of the world population. In addition, an increasing adoption of cancer-causing behaviors, particularly smoking in economically developing countries, also contributes to elevating the numbers of cancer diseases. The four most common cancers occurring worldwide are lung, female breast, colorectal and prostate cancer. These four accounts for around 4 in 10 of all cancers diagnosed worldwide\(^1\).

In our study, we mainly focused on breast and head/neck (oesophagus, hypopharynx) cancers. Breast cancer is the leading cause of cancer death among females, and it is also the most frequently diagnosed cancer, accounting for 23\% of the total cancer cases and 14\% of the cancer deaths. Oesophageal cancer is the eighth most common cancer worldwide (3.2\% of the total), and the sixth most common cause of death from cancer (4.9\% of the total)\(^2\).

The early diagnosis would be essential in all cases, to prevent further complications and the development of metastases. Unfortunately, in most of the cases, the specific symptoms occur only at the advanced stage, so the role of the screening programs and of the suitable tumour markers are high. The importance of the tumour markers should be also emphasized in the postoperative period, for early detection or exclusion of the recurrence of cancer or for the detection of a second tumour. There is a lot of attempts to find new markers, but still, it is very important to do researches on this field and improve the diagnostic tool for cancers.

Besides the early diagnosis and adequate therapy, reconstruction of the defects after oncological ablative surgeries is also a big challenge for the plastic-reconstructive surgeons. The purpose of these operations is mainly to improve the quality of life and restore the body image, without affecting the prognosis or detection of cancer’s recurrence. For example, the most important consequence of mastectomy is the psychosocial effect of the physical and aesthetic deformity, which can include anxiety, depression, and negative effects on body image and on sexual function\(^3,4\). Studies
suggest that breast reconstruction restores body image; improves vitality, femininity, and positively affects the patient's sense of well-being and quality of life.\(^5,6\)

The first reconstructive surgery in Europe was described in the 15th century. A Sicilian family of surgeons developed methods to repair wounds to ears and lips and to reconstruct nose defects.\(^7\) Later in Bologna, a surgeon called Gaspare Tagliacozzi, who wrote the first book devoted to plastic surgery, described the first delayed flap for nasal reconstruction, and he experimented with cutting flaps of skin, called pedicles, from one part of the body and sewing them to another. Although, the procedure was not performed regularly until the 1800s.\(^8\) The first successful skin graft was attributed to Sir Astley Cooper in 1817. Since then the technique improved a lot, and there are different opportunities for the reconstruction, depending on the affected area, and type of the tumour. One optional procedure is the reconstruction with autologous tissues, when the own tissue of the body is used for reconstruction. In these cases different flaps can be chosen: local flap, regional flap or free tissue transfer. Local flaps are created by freeing a layer of tissue and then stretching the freed layer to fill a defect. Regional or interpolation flaps are not immediately adjacent to the defect. Instead, the freed tissue, "island" is moved over or underneath normal tissue, to reach the defect, with the blood supply still connected to the donor site via a pedicle.\(^9\) This pedicle can be removed later on after new blood supply has formed, e.g: PMMC (pectoralis major myocutaneous), DP (deltoplectoral) flaps for head and neck defects, TRAM (transverse rectus abdominis muscle) for breast reconstruction.\(^10\) Free tissue transfer is defined as the vascular dissection and detachment of an isolated and specific region of the body (eg, skin, fat, muscle, bone) which is transferred to another region of the body. For this purpose, an anastomosis of the divided artery and vein to a separate artery and vein located at the site of the defect is required. The microanastomosis ensures the perfusion, drainage, and the survival of the flap. This ability to transplant living tissue from one region of the body to another has greatly facilitated the reconstruction of complex defects.

Free tissue transfer has become a routine procedure in many centers around the world. The numerous advantages of this technique include stable wound coverage, improved aesthetic and functional outcomes, minimal donor site morbidity, and the ability to utilize vascularized tissue from remote parts of the body that are outside the zone of injury (trauma, malignancy, infection, irradiation, etc). Since the introduction of free tissue transfer in the 1960s, the success rate has improved substantially, currently being 90-95% among experienced surgeons. Although the success rates of these
surgeries are high, there are still some cases, where the insufficient microcirculation, caused by ischemia-reperfusion injury (IRI), leads to partial flap loss and results in the reoperation of the patient. In addition, the flap/limb can become irremediable because its poor circulation, and it may make the reconstruction more difficult or impossible\textsuperscript{11,12,13,14,15}. For these reasons the detection of biochemical changes and microcirculatory disorders in flaps during ischemia-reperfusion (I/R), are of high importance\textsuperscript{16,17}.

1.1 ISCHEMIA-REPERFUSION INJURY (IRI)

Ischemia-reperfusion injury is a cascade of pathophysiological events, that can occur after the reperfusion of the tissues, exposed to prolonged ischemia and results in tissue damage. Regarding with free flaps, it is mainly responsible for the damages of the distal microcirculation and parenchyma of the flap and can lead to partial flap loss\textsuperscript{18}. Metabolic alterations such as capillary narrowing, leukocyte sequestration, neutrophil infiltration, dysfunction of endothelium, end-organ membrane dysfunction and enzymatic defects of mediators, generation of free oxygen radicals, activation and triggering of cytokines and chemokines, the role of the complement system and the involvement of the mitochondria can influence the severity of the IRI\textsuperscript{19,20,21}. During ischemia, the metabolism shifts towards the anaerobic, which results in a decrease in cell pH. To buffer this accumulation of H\textsuperscript{+} ions, the Na\textsuperscript{+}/H\textsuperscript{+} exchanger excretes excess hydrogen ions, which leads to a large influx of sodium ions\textsuperscript{22}. Ischemia also depletes cellular adenosine-tri-phosphate (ATP), which inactivates ATPases (e.g., Na\textsuperscript{+}/K\textsuperscript{+} ATPase, Ca\textsuperscript{2+} ATPase), reduces active Ca\textsuperscript{2+} efflux, and limits the reuptake of calcium by the endoplasmatic reticulum (ER), thereby producing calcium overload in the cell. These changes are accompanied by opening of the mitochondrial permeability transition (mPTP) pore, which dissipates mitochondrial membrane potential. This can result in further depletion of the ATP, irreversible oxidation of proteins, lipids, DNA, and can trigger cell-death pathways\textsuperscript{23,24}. Although prompt reperfusion restores the delivery of oxygen and substrates required for aerobic ATP generation and normalizes extracellular pH by washing out accumulated H\textsuperscript{+}, reperfusion itself appears to have detrimental consequences as well. The mechanism underlying reperfusion injury are complex, multifactorial and involve: (1) generation of reactive oxygen species (ROS) that is fueled by reintroduction of molecular oxygen when the blood flow is reestablished, (2)
calcium overload, (3) opening of the mPTP pore, (4) endothelial dysfunction, (5) appearance of a prothrombogenic phenotype, and pronounced inflammatory responses. The inflammation, induced by I/R, through the release of endogenous molecules from necrotic and injured cells, typically occurs in the absence of microorganisms, so it has been termed as a sterile inflammation. Inflammation is an important process required for tissue repair and regeneration through the clearance of dead cells and the release of growth factors and chemokines that induce cell proliferation and angiogenesis. In IRI, inflammation becomes excessive and the injury and repair become unbalanced with innate immune cells playing a critical role in mediating injury responses. All cells of the innate immune system, including neutrophils, monocytes, macrophages, dendritic cells (DC), and natural killer (NK) cells express specific receptors (pattern recognition receptors (PRRs)) and therefore contribute to a pro-inflammatory environment that is established following reperfusion. Additionally, non-immune cells such as endothelial and epithelial cells also express PRRs. Ligation of PRRs results in the induction of nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPK) pathways. As a result, pro-inflammatory cytokines and chemokines, including interleukin 1 (IL-1), IL-6, tumor necrosis factor α (TNFα), monocyte chemoattractant protein 1 (MCP-1) and IL-8 are induced. Major histocompatibility complex (MHC) and costimulatory molecules are also upregulated and promote the recruitment and activation of neutrophils in posts ischemic tissues. Neutrophil infiltration promotes leukocyte adhesion to postcapillary venules and subsequent emigration of the tissues, inducing microvascular barrier dysfunction through the release of oxidants and hydrolytic enzymes.

Fig.1: Major pathological events contributing to ischemic and reperfusion components of tissue injury. Modified from Kalogeris et al.
1.1.1 Trimetazidine

Trimetazidine (TMZ), is a well known anti-ischemic drug, which so far, clinically used only in cardiology, as an anti-anginal treatment. In the second and third study we used trimetazidine (10 mg/kg) against ischemia-reperfusion injury since it has many properties which can be effective against it:

- decreases fatty acid oxidation and stimulates glucose utilization (via the inhibition of the mitochondrial long-chain 3 ketoacyl CoA thiolase) leading to the production of adenosine triphosphate (ATP) with less oxygen consumption\textsuperscript{29,30}
- limits intracellular acidosis, reduces sodium and calcium accumulation into cells\textsuperscript{31}
- inhibits the production of deleterious lipid metabolites
- inhibits mitochondrial permeability transition pore opening and protects tissues from prolonged ischemia-reperfusion injury\textsuperscript{32}
- decreases cytolysis and membrane injury caused by oxygen free radicals\textsuperscript{33}
- attenuates the inflammatory response and reduces the rate of apoptosis expression\textsuperscript{34}

Furthermore, Devynck et al. investigated the effect of TMZ on the membrane in human platelets and found that TMZ reduced cAMP content and aggregation responses to collagen and ADP\textsuperscript{35}. TMZ is accepted as an agent without any hemodynamic activities, and mainly minor side effects (episodes of a headache) were mentioned in a few cases\textsuperscript{36}.

![Mechanism of mitochondrial anti-ischemic effects of trimetazidine](image)

**Fig. 2**: Mechanism of mitochondrial anti-ischemic effects of trimetazidine
2 **AIMS**

We planned to perform three major investigations. In the first study, we focus on a new diagnostic opportunity for breast cancers. In the second and third study, the possibilities of the reduction of ischemia-reperfusion injury during reconstructive free flap surgeries, are in the centre of interest.

1. In the **first study**, we aimed to investigate the role of BFSP1 protein, in human breast cancers. First of all, we would like to prove that BFSP1 proteins can appear not just in the eye lens, but also in the tissues of human breast cancers. We would like to examine, whether it is any difference between the normal and the tumorous breast tissue, in the contents of BFSP 1 protein or not, so we plan to perform Western-blot analysis and immunohistochemistry examination. We also would like to determine, whether it is any difference in the BFSP1 content in tissue samples of patients, who received different treatment, or not. (Preliminary study to create a reliable diagnostic kit for breast cancers).

2. The first aim of the **second study** is to demonstrate, that measurable injury caused by ischemia-reperfusion, occurring in the flaps before macroscopically visible changes (e.g.: tissue necrosis) have developed. Furthermore, our main aim is to investigate the effects of Trimetazidine on oxidative stress, inflammation, and histopathological alterations, using the epigastric skin flap model in rats. To determine the efficacy of TMZ, we would like to measure different oxidative stress parameters, such as the levels of blood malondialdehyde (MDA), reduced glutathione (GSH) and plasma thiol groups (SH-). To evaluate the degree of the inflammation we also would like to determine the tissue TNF-alpha levels. Histopathology, immunohistochemistry and hemorheological examinations are planned to carry out to confirm the results of the biochemical analysis. Furthermore, in this study, we would like to examine two different administration route of the drug (preischemic and postischemic), to determine which one is more effective in reducing ischemia-reperfusion injury in skin flaps.
3. In the **third study**, we aimed to investigate the effect of Trimetazidine in rat small intestine. Compared to the skin, the jejunum is much more sensitive for the ischemic insult. We decided to administer the same dose of Trimetazidine as we administer in the skin flaps and evaluate the effect. Moreover, we would like to compare the effect of Trimetazidine with the effect of ischemic pre- and postconditioning (IPre; IPostC) in reducing the ischemia-reperfusion injury. We also would like to investigate, whether there is an additive effect of the pharmacological (with TMZ) and the ischemic pre- and postconditioning, or not. The level of the oxidative stress will be follow up with the determination of the malondialdehyde (MDA), reduced glutathione (GSH) and thiol group (-SH) plasma levels and of the superoxide dismutase (SOD) enzyme activity. From the inflammatory cytokines, the level of TNF-alpha and IL-6 will be measured. To evaluate the visible changes in jejunum, in the investigated groups, histopathological (HE, TUNEL) investigations will be performed as well.
3 THE ROLE OF BFSP1 PROTEIN, IN PREDICTION OF (BREAST) CANCER

3.1 INTRODUCTION

The global importance of cancer is unquestionable, considered the second cause of death worldwide. Breast cancer (BC) is the second most common cancer overall and the most frequent type of cancer in women worldwide\(^\text{37}\).

BFSP1 (Beaded Filament Structural Protein 1, or Filensin) is an eye lens specific cytostkeletal protein, forms intermediate filaments (IFs) with its assembly partner (BFSP2; phakinin).

Intermediate filaments are major structural elements of cells that maintain the shape of cells and nuclei and regulate cell motility and adhesion, which in the context of the primate lens will include lens accommodation\(^\text{38}\). A wide range of inherited diseases is caused by mutations in intermediate filament proteins or their associated proteins as IFs are closely linked into intracellular scaffolding and transport machinery as well as important signaling pathways that determine cell survival and cell death\(^\text{39}\). There are around 70 different gene products attributed to the intermediate filament protein family. IF proteins are divided into six types based on their amino acid sequences\(^\text{40,41}\).

Intermediate filaments are dynamic cytoskeletal structures that are involved in a wide range of cellular processes during all life stages, from development to ageing, and in processes involving both stress and homeostasis. Furthermore, IFs form an extensive and elaborate network which connects the cell cortex to intracellular organelles and likely contributes its biophysical properties to the mechanical and motile properties of the cell. By playing an extensive role in cell migration IFs are responsible for tumour spreading where cancer cells invade adjacent tissues and form metastases\(^\text{42,43}\).

The expression pattern of IF proteins are tissue-specific and developmentally regulated. The expression of specific subsets of IF proteins classically serve as biomarkers to identify the tissue origin of the tumours. IF typing distinguishes the major tumor groups: carcinomas are characterized by cytokeratins, sarcomas of muscle cells by desmin, nonmuscle sarcomas by vimentin, and gliomas by glial fibrillary acidic protein. Therefore, the use of antibodies which are specific for one type of intermediate filaments can determine the histogenesis of tumours in certain cases, that are difficult to diagnose by conventional techniques\(^\text{44}\).
BFSP1 has been known as a cytoskeletal intermediate filament expressed exclusively in the eye lens so far. Although the biological functions of filensin and phakinin are not clear, evidence indicates that they play an important role in maintaining lens transparency during fetal development and fiber cell differentiation\(^{45,46}\).

Antal Tapodi, Daniel M. Clemens and co-workers examined the original role of the BFSP1 in the lens and have discovered that BFSP1 is expressed unexpectedly in human breast adenocarcinoma cell line (MCF7) as well as in human cervix carcinoma cells (HeLa) (under review). The appearance of BFSP1 in cancer cells seems very surprising and it indicates a new exciting approach in the field of tumour biology.

The gene of filensin is located on chromosome 20: 17,493,905-17,569,220. Alternative splicing of BFSP1 results in multiple transcript variants. Four splice variants of BFSP1 were detected so far, out of which the splice variant No. 1 is the eye lens specific. Compared to the splice variant No. 1, the splice variants No. 2, 3 and 4 are shorter lacking even longer part of the N-terminal head-domain respectively.

\[\text{Fig. 3.}: \text{Schematic representation of BFSP1 splice variants. Splice variant number 2 and 3 are overlapping, truncated versions of splice variant number 1, while number 4 has a different amino acid sequence at the N-terminal end, indicated with pink color on the figure. 433 and 549 are caspase cleavage sites on the tail domain of the protein.}\]
3.2 MATERIALS AND METHODS

Preliminary experiments of Antal Tapodi (PhD, Department of Biochemistry and Medical Chemistry, Medical School (MSch), University of Pécs (UP)) and his coworkers showed that BFSP1 is present in human-derived in vitro cultured tumorous cell lines, hence raising a question, if filensin can be found in human tumour tissue as well. In order, to ascertain our theory, we tested ex vivo human breast carcinomas. Our research was approved by the Regional Research Ethics Committee of the Medical Center, Pécs. (*Document number: 6446-PTE 2017/2018*).

3.2.1 Protocol

We started our study in April 2017, in cooperation with the Surgery Clinic, the Department of Pathology and Department of Biochemistry and Medical Chemistry. Since then, so far 25 patients were involved in this research. The only criteria for participating in the experiment were the existence of a diagnosed tumour and the signed declaration of agreement, regardless of gender, age, and type of carcinoma.

In this study, our research group examined particularly breast cancer-derived tumour samples, after mastectomies. The patients, who were involved in this study signed a declaration of agreement of this study at the Surgery Clinic (MSch, UP). After the surgery, the completely removed breast side was sent to the Department of Pathology (MSch; UP), where the pathologists first performed the histopathological examinations for the sake of the proper diagnosis and further treatment. Then, the sampling was performed for our study. A small amount of the breast tissue (both from the tumor and tumor-free area) was sent in RNA later solution to the Department of Biochemistry and Medical Chemistry (MSch, UP) for further investigations, such as mass spectrometry (MS), RNA examination and Western-blot analysis. Remained part of the breast tissue was evaluated by the same pathologist, under the microscope at the Department of Pathology. He performed slices from the tumour, and from tumour-free area. After the adequate preparations of the slices, they were incubated overnight at 4 °C in the presence of the primary antibody. (The exact method is written down below: 3.2.6: „Immunohistochemistry”)

In this study, only the results of the Western blot analysis and immunohistochemistry are involved. For the Western blot analysis, we examined
tumorous and a non-tumorous (from behind the nipples) breast tissue parallelly, to make sure if the filensin is present only in tumour cells. In the Department of Biochemistry (MSch, UP), the samples were homogenized and immunoprecipitated to eliminate unnecessary contamination, and then, the eluted samples were lyophilized and examined with Western Blot.

3.2.2 Primary antibodies

We used two different primary rabbit polyclonal antibodies which were raised against various parts of the BFSP1 protein to allow us the detection of the different proteolytic fragments of BFSP1 as well. The S38 antibody is anti-BFSP1 (HPA042038 Sigma) antibody, which is capable to recognize both major proteolytic fragments. The S48 is the anti-BFSP1 antibody (HPA040748 Sigma) raised against the N-terminus proteolytic fragment of BFSP1.

![Fig. 4: The full-length sequences of the BFSP1 protein. With orange color, the recognition sequence of the S48, with blue color the recognition sequence of the S38 antibody are marked.](image)

3.2.3 Lysis and Homogenization

The frozen tumour and non-tumour samples were thawed, then cut into pieces (approx. 5mm) with scissors. Afterward, the tissue was ground with tissue grinder (IKA ULTRA-TURRAX Homogenizer T-18) in a lysis buffer (8M urea, 20mM TRIS-HCl pH=8.0, 1mM EDTA, 150 mM NaCl, 0.5% Triton® X-100, 1 tablet of protease inhibitor
cocktail, dH₂O up to final volume), then sonicated with ultrasound. After centrifugation (16,000 x g) the supernatant was separated from the pellet and the occasional lipid layer and transferred to clean tubes. In order to remove urea and gain clean proteins, we performed a chloroform-methanol total protein precipitation. This was achieved by mixing 1-part sample with 4-part 100% methanol, 1-part 100% chloroform and 3-part dH₂O. After centrifugation (16,000 x g) the supernatant had been removed, the pellet was washed with 7-part 100% methanol to remove the chloroform below the pellet. Following a centrifugation step (16,000 x g) the pellet was resuspended and re-sonicated in lysis buffer for further examination.

3.2.4 Magnetic purification, lyophilization

The magnetic immunoprecipitation procedure happened to be the same for the tumour, non-tumour lysates.

We used BioMag® Goat anti-Rabbit IgG (Cat. No. 84300), magnetic agarose beads, conjugated covalently with secondary antibody. To fix the beads we applied a Qiagen 12-Tube Magnet rack (Cat. No. 36912).

200ml of bead suspension was taken into clean tubes for each sample. The magnetic beads were fixed in the wall of the tube towards the magnet in approx. 3 minutes. The containing buffer had been removed, the beads were washed with PBS 3 times. Afterward, the diluted S38 primary antibody (1:500 in 3% BSA in TBS) was incubated with the magnetic bead – secondary antibody complex for 1 hour in room temperature while shaking. After removal of the buffer, 1ml of each tumor – non-tumor lysate and 700µl of each serum sample was incubated with the previously bound complex for 2 hours in room temperature while shaking.

The immunoprecipitated complex was washed 1 time with Buffer 1 (20 mM TRIS pH=7.5, 150 mM NaCl, 1% Triton® X-100); 2 times with Buffer 2 (20 mM TRIS pH=7.5, 150 mM NaCl); and 2 times with Buffer 3 (5 mM TRIS pH=7.5). After the washing steps, the elution was done by 1M NH₄OH, to avoid the possible contamination.

The eluted samples were frozen to -80°C, then lyophilized with Christ® Alpha 1-2 lyophilizer overnight in order to eliminate the solvent. The solid immunoprecipitated
samples were re-dissolved in 5x Sample buffer [0.25 M Tris-HCl, pH: 6.8; 0.5 M DTT; 10% SDS; 50% Glycerol; 0.5% bromophenol blue].

3.2.5 Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% gel, with Bio-Rad® Mini-PROTEAN Tetra cell with the current of 120 mV in 1X Running Buffer (250 mM Tris, 1.92 M Glycine, 1% SDS). The run proteins were then transferred to nitrocellulose membrane for blotting using semi-dry method. After the visualization with Ponceau dye, the remaining membrane surface was blocked by 10% milk powder and 6% BSA in TBS at room temperature, for 2 hours. The membranes then were welded into plastic bags, together with 1 ml of the primary antibody, S38 or S48, respectively, diluted in 1:500 ratio in 3% BSA – TBS for overnight at 4°C. Afterward, the nitrocellulose membranes were placed into secondary antibody (EIA Grande Anti-Rabbit IgG Horseradish Peroxidase Conjugate, Cat. No. 172-1019) diluted in 3% BSA – TBS, incubated for 2 hours in room temperature. Finally, the membranes were visualized with ImmunoCruz Western Blotting Luminol Reagent, at the Szentágothai Research Center with a Fujifilm LAS 4001 multipurpose CCD camera system. The detected images were merged with the molecular weight marker with Adobe Photoshop CC 2018.

3.2.6 Immunohistochemistry

Human breast tumour tissues were fixed in 4% buffered formaldehyde. Dehydration was performed with Leica automatic instrument. Tissues were embedded in paraaffin, slices were cut with a thickness of 3-4 μm and put onto silanized slides. Deparaffination was performed three times for 5 minutes in xylene, following three times 5 minutes washing with ethanol in decreasing concentration (90%-70%-50%). Then the samples were washed in distilled water for 3 minutes. Antigen retrieval was performed in citrate buffer pH=6.0 three times for 5 minutes. Samples were microwaved for one minute at 750 W. Then the samples were washed in 0.5 M Tris buffer pH=7.6 three times for 2 minutes. Inhibition of endogen peroxide was achieved in 3% hydrogen peroxide for 10 minutes at room temperature. Samples were washed in 0.5 M Tris buffer pH=7.6 three times for 2 minutes and then were blocked in 1 % horse
sera diluted in 0.5 M Tris buffer pH=7.6. Slices were dried on air and were incubated overnight at 4 °C in the presence of primary antibody. (S38 and S48, 1:200 dilution) diluted in 1% horse sera in 0.5 M Tris buffer pH=7.6. Visualization was made with EnVision™ FLEX, High pH (Dako Autostainer/Autostainer Plus). Background staining was according to Mayer’s Hematoxylin staining for 3 minutes. Rehydration was performed in increasing concentration of ethanol (50%-70%-90%), then samples were washed in xylene three times for 5 minutes. Finally, the slices were covered with Pertex Mounting Media (Leica Biosystem).
3.3 RESULTS

3.3.1 Western blotting

Preliminary data has shown that filensin appears in “in vitro” cultured human tumour cell lines. Following this logic, we examined human ex vivo clinical samples, precisely breast cancer tumour samples from cancer patients.

In this study, we have proved with immunoblotting, that BFSP1 is present in tumour samples (T1, T2, T3). The non-tumour tissue was used from the same person (mainly from behind the nipple-areola complex) as a control. We proved the absence of filensin within the normal tissue with both types of antibodies. (N1, N2, N3).

Fig 5. Western Blot analysis of human ex vivo breast cancer samples performed by two primary antibodies, S38 and S48, respectively. In the case of S48, the top arrow shows the native BFSP1, while the bottom arrow shows a proteolytic fragment of the protein, similarly to the S38 membrane.

As a pilot experiment, we performed Western blot from lyophilized tumour and serum samples as well, from the same individuals, hence proving that the BFSP1, which we identified, is the same in both types of samples. This provides a good background for our further investigations with serum.
Fig. 6.: This western blot image represents lyophilized tumour and serum samples run simultaneously, proving that BFSP1 is present in both samples, and shows similar electrophoretic behavior.

3.3.2 Immunohistochemistry

For the identification of BFSP1 protein, immunohistochemistry was performed with the S38 and S48 anti-BFSP1 antibody. We are presenting our results from three patients according to their therapy. Samples with the same letter belong together and come from one patient. Letter A means: the patient did not receive neoadjuvant therapy and the tumour was presented. Letter B samples are from a patient, who received neoadjuvant therapy before the surgery but the tumour is still presented. Letter C represents a patient who received neoadjuvant therapy and the tumour was regressed.

Fig. 7. shows those results which come from the tumour-free area of the different treated patients. Here the structures are kept, we can identify the followings: adipose tissue, lobules and the stroma (interlobular/intralobular). Here only the ducts, ductules and acini were stained positively with the antibody, but there were no positive cells in the surrounding tissues. The different treatment did not have an effect on the number of positive staining cells in this area. The results are the same with the S38 and S48 antibody.

Fig. 8. shows those areas where the tumour was found or where the tumour was described before the neoadjuvant therapy. In the case of the S38 antibody strongly positive staining cells can be seen on the first and second pictures, where the tumour was presented. (Fig. 8/A, B). The positivity is not well-defined and more extensive than in Fig. 7. Picture C on Fig. 8, shows significantly less positive staining cells than the other pictures. This patient got neoadjuvant therapy, and after this, the macroscopical tumour could not be detected. This can be the explanations of the reduced amount of
the positive cells. According to our results so far, BFSP1 protein can be a sensitive marker in the case of ductal carcinomas.

**Fig.7.:** Immunohistochemistry with S38 and S48 anti-BFSP1 antibodies. (10x). These pictures are from the tumour-free area (behind the nipple). Every picture represents another treatment method. (Description is in the text). Here only the ducts are stained positively with the antibody.

**Fig.8.:** Immunohistochemistry with S38 and S48 antibodies. (5x) These pictures are from the tumour or from that area where the tumour was. Every picture represents another treatment method, and the pictures on Fig.7. and on Fig. 8. with the same letters are from the same patient.
Comparing the results of the S38 and S48 antibodies, we found stronger positivity in those cases where the S38 antibody was used. One possible explanation for this, is the different binding and recognition sites, the S48 antibody is bound to the membrane and recognise only one proteolytic fragment of the protein, while the S38 shows up in the cytoplasm (Fig. 9) and recognise both proteolytic fragments. Since the positivity in the tumour cells was stronger with S38 antibody, it suggests that the proteolytic fragment after the D433 caspase cleavage site has stronger relation with the presence of the tumour cells.

Fig. 9: These pictures show the results at greater magnification (20x). It is clearly visible that the S38 antibody shows up in the cytoplasm (Picture A), while the S48 antibody is bound to the membrane (Picture B).
3.4 DISCUSSION

The global importance of cancer is unquestionable, considered the second cause of death worldwide. Breast cancer (BC) is the second most common cancer overall and the most frequent type of cancer in women worldwide\textsuperscript{37}. For routine surveillance and for staging mammography and ultrasonography are commonly used. There are also different BC markers which can help to predict the prognosis or to select the suitable therapy. The most common BC markers are the estrogen receptor (ER) and the progesterone receptor (PR) status. The absence of these receptors is a predictor of a poor prognosis. Furthermore, today, they are also used and suitable to select hormone therapy\textsuperscript{47}. There are several well-established serum markers; as the cancer antigen (CA) 15-3 (MUC-1 antigen) and carcinoembryonic antigen (CEA) levels, which are determined at diagnosis of systemic recurrence. However, they do not increase in all patients; a recent study showed increased CA 15-3 and CEA levels in only 55.6 and 36.0\% patients at diagnosis of systemic recurrence, respectively\textsuperscript{48}. Moreover, these markers are used to help for the detection of distant metastases, however, they have limited value in diagnosing micrometastases or locoregional recurrences. Several benign diseases, as well as chemotherapy, seem to influence their levels so they also suffer from a lack of cancer specificity\textsuperscript{49}.

Therefore, the identification of markers that could predict tumor behavior is particularly important in breast cancer, since the determination of tumor markers is a useful tool for the clinical management of cancer patients, assisting in diagnostic procedures, staging, evaluation of therapeutic response, detection of recurrence, distant metastasis and prognosis, helping in the development of new treatment modalities.

The cytoskeleton comprises three major filament systems — microfilaments, microtubules, and intermediate filaments (IFs), and collectively, these provisions and maintain cell shape and structure, and are key to important cellular events, including cell division, movement, and vesicular transport. IFs can be formed from 40 different subunit proteins. The different types of IFs can be distinguished according to their localization and protein composition. Intermediate filaments are expressed in various cells with determined specificity. Due to this phenomenon, IFs can be used as indicators determining the origin of protein based on the tissue-specific expression pattern in such cells. IF typing is also a method in cancer diagnosis because of the above-mentioned properties.
Beaded Filament Structural Proteins (BFSPs) belong to the family of intermediate filament proteins (IF). BFSP1 or filensin is expressed in lens fiber cells after differentiation has begun. Although their role in lens biology has still not been clearly defined, these intermediate filament (IF) proteins are essential to the optical properties of the lens\(^\text{50}\). They are also important to its biomechanical properties, to the shape of the lens fiber cells, and to the organization and function of the plasma membrane\(^\text{51}\). The critical role of these proteins is mainly emphasized by the presence of cataracts\(^\text{52,53,54}\).

Antal Tapodi and coworkers previously examined the biological role of BFSP1 in the eye lens. Originally, they were about to determine the caspase cleavage events of the endogenous filensin protein. Achieving this, they cloned and expressed recombinant BFSP1 in human, commercially available cell lines, namely MCF7, a breast carcinoma derived- and HeLa, cervical cancer-derived, widely used cell lines. While visualizing their results via western blot, however, an extra band was observed in the untransfected, negative control cell lines as well. This surprising discovery raised many further questions since BFSP1 was only known as an eye lens specific intermediate filament protein. In 2014 they also analyzed six commercially available cell lines with western blotting, namely: U-118 MG, U-251 MG (glioblastoma cell lines), A-549 (human lung cancer), T24/83 (human bladder carcinoma), HeLa and HepG2 (human liver cancer), and have proven that BFSP1 is present in each of them.

In this study, we continued an ongoing project examining the unexpected presence of BFSP1 protein in tumour cells. This is the first study, where the expression of BFSP1 was demonstrated in ex vivo tumour samples and serum as well (Figure 4, 5). Furthermore, I would like to emphasize, that with Western-blot analysis this protein was presented only in the tumour samples, and we proved the absence of filensin within the normal tissue.

With immunohistochemistry, using the S38 antibody, we could confirm that there is a significant difference in the contents of BFSP1 according to the presence of the tumour. In those patients, who received/did not receive neoadjuvant therapy but/and the tumour was presented macroscopically, the number of the positive staining cells increased considerably, compared with the tissue samples of those patients who received neoadjuvant therapy and the tumour was regressed. In the case of the S48 antibody our results were not so convincing. The explanation of this can be the different recognition sequence of the two antibodies. Based on our results it seems that certain
part of the BFSP1 protein can be sensitive enough to indicate the tumour cells in the case of ductal breast carcinomas, but to understand the role of the protein further investigations are needed.

It is very important to note that, this study is part of a greater project, therefore our research group has numerous plans according to the future. We are planning to continue examining the human ex vivo clinical samples, including benign breast tumours, other types of breast cancers, and other types of cancers, like melanoma and colorectal cancers. Furthermore, we would like to perform a quantitative real-time PCR analysis from ex vivo clinical samples as well as mass spectrometry. Investigation of the pre- and postoperatively (on the 1st and 3rd week) collected serum, to assess the possible quantitative changes of the BFSP1 protein after the surgery, is also included in our future plans.

On the other hand, the biological role of BFSP1 in tumour cells is yet unknown. One of the major goals of our research team is, to identify the possible interacting proteins co-precipitated with native BFSP1. Antal Tapodi and his co-workers proved previously different intracellular distribution of the proteolytic fragments of BFSP1, so we suppose that BFSP1 fragments might play a different role in tumour cells. Finally, we can say that the major goal of our ongoing research is to create an affordable diagnostic tool, which could be used in daily medicine, helping cancer patients in a time of need.

3.5 CONCLUSION

As a conclusion, so far we can say, that BFSP1 protein is expressed not just in the eye lens but also in human breast cancers. We examined 25 patients with ductal carcinomas in this study. With immunohistochemistry, we proved that BFSP1 protein shows sensitivity for the tumour cells, independently that the patients received neoadjuvant therapy or not. Furthermore, the same type of the protein is presented in the serum as in the tissue samples. This study provides a good base for further investigations which can specify the exact role and the type of splice variant of the BFSP1 protein involved in cancers, and which can study the presence of this protein, in a different type of cancers. In the case of ductal carcinomas, the protein can have an important role after the surgery in the follow-up period of the patients, and it could also be able to provide an adequate information about the recurrence of cancer from the serum of the patients, although additional studies are also required in this field.
4 THE EFFECT OF TRIMETAZIDINE IN REDUCING THE ISCHEMIA-REPERFUSION INJURY IN RAT EPIGASTRIC SKIN FLAP

4.1 INTRODUCTION

Ischemia-reperfusion injury (IRI) can cause considerable problems in various fields of the surgery, like in reconstructive plastic surgery, vascular surgery, traumatology or cardiac surgery. IRI is a cascade of pathophysiological events, that can occur after the reperfusion of the tissues, exposed to prolonged ischemia and results in tissue damage. Unfortunately, this condition is unavoidable during free flap surgery or during replantation. Free tissue transfer has become a routine procedure to cure tissue defects after oncological ablative surgery or trauma. In the last decade, the technique of the free flap surgeries improved a lot and it has reached the 90-95% success rate. Although the success rates of these surgeries are high, there are still some cases, where the insufficient microcirculation, caused by IRI, leads to partial flap loss and results in the reoperation of the patient. In addition, the flap/limb can become irremediable because its poor circulation, and it may make the reconstruction more difficult or impossible. For these reasons, the detection of biochemical changes and microcirculatory disorders in flaps during I/R, are of high importance.

Even though many drugs and methods have shown promising results experimentally, there hasn’t got any existing consensus treatment in the clinical practice, because of their unfavourable systemic side effects, excess toxicity, limited efficacy, invasive administration or because of the time-consuming technique.

Trimetazidine (TMZ, water-soluble form: trimetazidine-dihydrochloride) is a widely used anti-anginal drug worldwide. It is a potent anti-ischemic agent and a free radical scavenger. It has been used in many studies to protect different organs (myocardium, intestine, liver, and kidney) from the ischemia-reperfusion injury. Numerous evidence exists, which shows that the reperfusion injury could be decreased by TMZ-preconditioning in animals. It was found that TMZ conserves ATP production, maintains cellular homeostasis and reduces the intracellular acidosis. Moreover, it decreases the oxidative damage to the mitochondria and protects the organ from tissue damage, induced by IRI.
According to the previous studies, we believe that a single shot of TMZ will be protective against IRI also in our study. This study aimed to investigate the effect of trimetazidine on oxidative stress, inflammation, and histopathological alterations (before visible changes (e.g. tissue necrosis) occur), using the epigastric skin flap model. To determine the efficacy of TMZ, levels of blood malondialdehyde (MDA), reduced glutathione (GSH), and plasma thiol groups (SH-) and tissue TNF-alpha were measured, histopathology and immunohistochemistry were performed.

4.2 MATERIALS AND METHODS:

4.2.1 Animal model

Forty male Wistar rats of the same age, weighing between 350 to 400 g, were used for this study. The rats were housed in separate cages, under standard conditions (temperature: 25±2 °C, and air filtered room), with 12/12-hour light-dark regimen and were fed with standard rat chow and water ad libitum. Food was withdrawn 12 hours prior to the experiment. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation. (number: ZOHU0104L 16)

4.2.2 Experimental protocol

The animals were divided randomly into four groups (10 rats in each group). The first group was the non-ischemic control group. Although the control flaps did not undergo ischemic insult, flap harvest may produce some temporary ischemia. In the other groups (groups 2 through 4) ischemia was induced by placing a single microvascular clamp across the epigastric superficial artery and vein. In the second group (I/R) the superficial epigastric vessels were clamped for 6 hours, followed by 24 hours of reperfusion. The third (Preisch.TMZ + I/R) and fourth (I/R+Postisch.TMZ) groups were the trimetazidine treated groups. In the third group, the TMZ was administered 30 minutes prior to the ischemic period. In the last group, animals received the drug at the onset of the reperfusion (Fig. 10.). To standardize the study, all procedures were performed at similar time points in all groups. Animals, in the treated groups, received 10 mg/kg trimetazidine (trimetazidine-dihydrochloride, Sigma-Aldrich, St. Louis, Missouri, USA) intraperitoneally (i.p) depending on the groups, 30
minutes prior to ischemia (Preisch.TMZ+I/R) or at the onset of the reperfusion (I/R+Postisch.TMZ). The drug was freshly solved into 0.9 % NaCl solution before the administration.

Fig. 10.: Investigation groups: Group 1: Control, Group 2: Ischemia-reperfusion (I/R), Group 3: Preisch. TMZ + I/R, Group 4: I/R + Postisch. TMZ

4.2.3 Surgical procedure

The rats have perioperatively anesthetized with an intraperitoneal (i.p) application of a mixture consisting of ketamine hydrochloride (5 mg/100g) and diazepam (0,5 mg/100g). The ratio was 1:1. The skin of the abdomen was depilated using an animal depilatory agent. During the operation, the animals were placed on a heated pad and ECG monitoring was also used. The carotid artery was catheterized (22 gauge) for blood pressure measurement. (Siemens Sirecust 1260, Düsseldorf, Germany). The skin of the abdomen was scrubbed with betadine and then 3x6 cm flap was created on both sides of the abdomen. In our study, the epigastric flap was chosen, because it simulates microsurgical free tissue transfer closely. This model was first described in 1967 by Strauch and Murray and has been widely used in various experimental animal researches on IRI and skin flap survival\textsuperscript{19,67,68}. The flaps include the area within the boundaries of the costal arch as an upper limit, the inguinal ligament as a lower limit
and both axillary lines as lateral borders. The medial borders were on both sides of the midline structures (the xiphoid and pubis). The vascular supply of the flap is provided by the medial and lateral branches of the superficial epigastric artery and accompanying veins, based on the superficial epigastric vascular pedicle. After 6 hours of ischemia, the microvascular clamp was released, and the blood flow was confirmed by arterial pulsation, flap colour, and vascular patency tests was also performed to ensure that the blood flow is recovered successfully. Flaps, where we could not detect any flow, were not included in this study. After checking the blood flow, the skin was sutured back to its original place with interrupted stitches (5-0, Prolene® (Ethicon), 30 stitches on both flaps). After the operation, the animals got a collar neck to prevent the automutilation. On the next day, before the sampling, animals were re-anesthetized.

Skin samples (3x1 cm) were taken from the most distal end of the flaps, after 24 hours of reperfusion, for biochemical examination. The samples were stored immediately at -80 °C within individual containers.

4.2.4 Biochemical analysis

MDA, GSH, SH levels were measured from the blood. MDA is a marker for the quantification of membrane lipid peroxidation. MDA levels were detected using a photometric method of Placer, Cushman and Johnson69. GSH and plasma SH levels were determined in anticoagulated whole blood by Ellman’s reagent, according to the method of Sedlak and Lindsay70. Both indicate the antioxidant status of the body.

To measure the TNF-alpha levels, samples were taken from the central part of the flap. Tissue TNF-α (one of the indicators of the inflammatory response) levels were studied by using the Rat TNF-α ELISA Kit (Abcam, Cambridge, UK) following the manufacturer’s protocol.

4.2.5 Histopathological analysis

A histopathological study of the samples was carried out by the same pathologist. The tissue samples were fixed in 4% neutral buffered formaldehyde solution and embedded in paraffin. Three-micron-thick (Microtome: Thermo Scientific Microm Hm 325) histological sections were cut, mounted on glass slides, stained with hematoxylin-eosin (HE) and evaluated by light microscope to quantify foreign body giant cells,
polymorphonuclear, and mono-nuclear reactive cells. For detection of apoptosis, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) was also performed.

4.2.6 Hemorheological analysis

4.2.6.1 Red blood cell deformability

In our study, we measured red blood cell deformability with LORCA ektacytometer. The blood sample was suspended in a high viscosity (28-32 mPas) polyvinylpyrrolidone solution and injected into the gap between the two cylinders. A laser beam transversing the suspension creates a diffraction pattern on a diaphragm that is recorded and analyzed by a video camera and a computer controlled ellipse fitting. At rest, red blood cells show circular diffraction pattern and parallel to the applied shear stress it becomes elliptical as cells deform and elongate.

For analysis, elongation index (EI) was calculated as the (length - width) / (length + width) of the pattern for each shear stress (SS) at 9 different shear stresses (from 0.3 to 30 Pa). For data analysis, the Lineweaver-Burke nonlinear curve fitting technique was used to calculate the maximal EI (Emax) value at extrapolated infinite shear and the shear stress value required for half of Emax (SS1/2). EI ranges from 0 to 1, 0 refers to an undeformed, randomly oriented RBC, and EI increases with cell deformation. Higher Emax refers to higher deformation ability, while RBCs with higher SS1/2 are harder to deform. Measurements were performed at 37°C. This micro-rheological parameter is an important determinant of the microcirculatory pattern.

Blood samplings were performed from lateral tail veins, and hemorheological examination was carried out before the surgery and on the 1st, 4th and 7th postoperative days, under anesthesia.

4.2.7 Statistical analysis

For statistical evaluation, one-way analysis of variance (ANOVA) was used, followed by adequate post hoc tests (Dunnett’s, Sidak) for multiple comparisons. All data are represented as the mean ± SEM. The difference was considered statistically significant when the p-value was less than 0.05.
4.3 **RESULTS:**

4.3.1 **Changes of oxidative stress parameters in blood samples**

4.3.1.1 **Malondialdehyde**

The statistical analysis of the MDA levels showed reduced values both in pre-and postischemic trimetazidine treated groups compared to the I/R group, however, a significant decrease was shown only in that group where the TMZ was administered prior to the ischemia (59,84±2,8 vs. 75,3±6,4; p=0,0145). These results refer to smaller lipid peroxidation in the treated groups. MDA levels were considerably higher only in the I/R group, compared to the control (75,3±6,4 vs. 50,85±1,4; p<0,0001). The results of the treated groups were nearly as good as in the control group. (Fig. 11)

![Graph showing MDA levels](image)

**Fig. 11.** Malondialdehyde concentrations in the experimental groups. MDA serves as a marker of the lipid peroxidation. *: p<0,05 vs. control; #: p<0,05 between the signed groups; error bars: SEM
4.3.1.2 **Reduced Gluthatione**

GSH levels were reduced in all groups comparing to the control (Control: 1116±38,09). Significantly higher GSH levels were measured both in pre-and postischemic trimetazidine treated groups (preisch. TMZ: 965,5±6,3, p=0,0035; postisch. TMZ: 1002±38,6, p=0,0002 vs. 820,9±13,5) compared to the I/R group, which supported the antioxidant effect of the drug. (Fig.12)

![Bar chart showing plasma concentration of reduced glutathione (GSH) in different groups.](https://via.placeholder.com/150)

**Fig. 12:** Plasma concentration of reduced glutathione in the investigated groups. GSH serves as a marker of the antioxidant status. *: p<0,05 vs. control; #: p<0,05 among the signed groups; error bars: SEM
4.3.1.3 Sulfhydryl group (SH-)

There were no significant differences in the SH- levels among the groups (control: 94,03±8,584; I/R: 74,3±3,763; preisch.TMZ: 98,62±11,4; postisch.TMZ: 91,65±6,5). (Fig.13)

**Fig.13.** Concentrations of SH- groups in the plasma. The levels of SH- refer to the antioxidant status. Error bars: SEM
### 4.3.2 Changes of TNF-α level in skin samples

Comparing to the control group, except the Preisch. TMZ+ I/R group, significantly higher values were measured (Control: 35093±4640). Considerable decrease of TNF-α levels in the treated groups were noticed, compared to the I/R group (preisch. TMZ: 41243±2183, postisch. TMZ: 54025±5924, p=0.0437 vs. 73331±5762), which can prove the anti-inflammatory effect of the drug. (Fig. 14)

![Graph showing TNF-alpha concentrations](image)

**Fig. 14.**: TNF-alpha concentrations show the grade of the inflammatory response in the investigated groups. *: p<0.05 vs. control; #: p<0.05 among the signed groups; error bars: SEM
4.3.3 Histopathological results
4.3.3.1 Hematoxylin-eosin

Our histopathological findings correlate with the biochemical results. Four zones are identified in all tissue samples. (Fig. 15, Control) In the control group, the basic tissue structures mainly kept, oedema, necrosis or significant inflammation cannot be detected.

In the I/R group (Fig. 15, I/R) many changes can be noticed: oedema was occurring in the fatty zone and in the submuscular zone. A large number of polymorphonuclear (PMN) cells could be seen under the muscle. The muscle fibres were swollen and irregular-shaped.

In both TMZ treated groups significantly fewer tissue changes were seen than in the I/R group. The muscle fibres were approximately normal shaped, oedema and PMN-cells were barely detected in the different zones. (Fig 15; Preisch. TMZ+I/R, I/R+Postisch. TMZ)

Fig. 15.: Staining: HE, magnification: 5x. In the control group, the four zones can be clearly identified: A: epidermal-dermal zone; B: fatty zone; C: muscular zone (B+C=panniculus carnosus), D: submuscular zone. In the I/R group oedema can be seen in the submuscular and fatty zone and the muscle fibres are swollen and irregular-shaped in the zone C. The protective function of the TMZ is well demonstrated in both (Preisch. TMZ+I/R and I/R+Postisch. TMZ) groups, showing less changes in the tissue samples: muscle fibres are approximately normal shaped, oedema and PMN-cells are barely detected in the different zones.
4.3.3.2 TUNEL-staining

The good influence of the drug is also supported by TUNEL staining (Fig 16). TUNEL-positive nuclei were stained brown. In the control group, the high number of positive cells are physiological, because they are showing up only in the follicle of the skin and these are holocrine glands. (Fig.16; Control). In the I/R group (Fig.16, I/R) many apoptotic cells were found in every zone of the flap. This confirms that I/R also promotes the apoptosis. The TMZ management of skin flaps clearly decreased the quantity of the apoptotic cells. Apart from the epidermal-dermal zone, where apoptotic cells can be found physiologically, the number of the positive cells were considerably fewer in the treated groups, compared to the I/R group. (Fig 16; Preisch. TMZ+I/R, I/R+Postisch. TMZ)

![Fig.16: Staining: TUNEL, magnification 10x: TUNEL staining demonstrates the apoptotic nuclei. 1. In the control group the high number of positive cells, showing up only in the follicle, are physiological - since these are holocrine glands. 2. The homogeneous positivity in the I/R group is the evidence to demonstrate the damage in the tissue, caused by the ischemia/reperfusion. 3. The protective function of the TMZ is well demonstrated in both (Preisch. TMZ+I/R and I/R+Postisch. TMZ) groups, showing barely positivity in all investigated zones.](image-url)
4.3.4 Changes in hemorheological parameters

**Figure 17.** illustrates the red blood cell deformability changes. The curve shows the elongation index (EI) of the red blood cells in the function of shear stress (SS). The preoperative and 7th postoperative days parameters did not differ; at most of the shear stress values the parameters were overlapping. However, on the 1st and mainly on the 4th postoperative day, the red blood cell deformability values were markedly worsened, dominantly in the I/R group.

**Fig. 17.:** Deformability of red blood cells in different groups preoperatively (Base) and on the 1st, 4th, and 7th postoperative days. The graph shows the elongation index (EI) of the red blood cells in the function of shear stress (SS)
4.4 DISCUSSION

The use of microvascular flap tissue transfer is very popular to reconstruct the defects of the whole body. It is known, that the success rate of the microsurgical vascular anastomosis, even with experienced surgeons is 90 to 95 percent, however, some severe problem such as IRI and the inadequate blood perfusion may still impede the complete success. IRI can cause severe problems in the microcirculation and it may lead to patient’s morbidity and prolonged hospitalization. The intracellular biochemical changes that occur during the ischemic period can cause cellular dysfunction, cellular and interstitial edema and finally can lead to cell death. The severity of these changes depends on the length of the ischemic time since it is well known that brief ischemic condition can be protective against the negative alterations\textsuperscript{74}. During reperfusion, following the ischemic period, reactive oxygen species are produced, which include oxygen ions, free radicals, and peroxides, all of which worsen ischemia-reperfusion damage\textsuperscript{75,76}, impact on red blood cells micro-rheological parameters and may result in considerable disturbance of blood flow\textsuperscript{77,78,79}. In the pathogenesis of I/R injury inflammation is also considered to be a critical element\textsuperscript{80,81}.

In our study, we chose the superficial epigastric skin flap model, because it was suitable to simulate a clinical situation, that occurs when microsurgical tissue transfer is made. As Yoshida and Campos suggested the model could also simulate a vascular pedicle thrombosis, where the procedure from the diagnosis to the restoration of blood supply could reach or exceed 6 hours, or it also can simulate a traumatic situation when replantation of amputated fingers is made\textsuperscript{82}. In these types of models, flaps contain the epidermal-dermal zone, fatty zone, muscular zone (panniculus carnosus) and submuscular zone with a vascular pedicle of the superficial inferior epigastric artery and vein. There are controversies related to the position of the microvascular clamp. They could be used both on the artery and on the vein, or separately on the vein or on the artery to simulate different situations, which can occur in the clinical practice. Our experimental model based on superficial inferior epigastric artery and veins to reach a higher level of I/R injury. The extension of the flaps was 6.0 x 3.0 cm bilaterally.

The length of the ischemic time was based on the literature\textsuperscript{83}; ÇetIn et al.\textsuperscript{78} subjected the rats to 6 hours and 10 hours of ischemia, because these time points have been reported to produce consistent biochemical, histopathological and macroscopic findings\textsuperscript{84}. 
TMZ is a potent anti-ischemic drug, which decreases fatty acid oxidation and stimulates glucose utilization via the inhibition of the mitochondrial long-chain 3 ketoacyl-CoA thiolase, leading to the production of adenosine triphosphate (ATP) with less oxygen consumption. It limits intracellular acidosis, decreases sodium and calcium accumulation into cells, inhibits the extracellular leakage of potassium during cellular ischemia and reduces cytolysis and membrane injury caused by oxygen free radicals. In addition, TMZ conserves mitochondrial function and energy metabolism and it is capable of inhibiting platelet adhesion-aggregation and neutrophil infiltration. Because it does not have a negative alteration on the hemodynamic status, besides the cardiology, probably it can also be useful in other areas of the clinical practice.

Previously, the effect of the TMZ on the survival of skin flaps was already studied and the agent was proved to be effective. Nieto et al. investigated various pharmacological agents on the survival of skin flaps in rats. All treated groups showed a significantly greater survival of the flap than the control group. One of the best outcomes was shown in those groups receiving trimetazidine and hydralazine. Kara et al. studied the effect of trimetazidine on the survival of rat island skin flaps. They compared the pre-ischemic and post-ischemic effect of the drug, and both ways seemed to be effective to improve flap survival.

However, this is the first study where, before the visible tissue changes, the histological and biochemical alterations were investigated after pre- and postischemic TMZ treatment in skin flaps. Blood MDA, GSH, and SH- levels and tissue TNF-α levels were evaluated for biochemical analysis. MDA is a stable product of polyunsaturated lipid peroxidation in cells, that is generated after free radical damage. GSH is one of the major endogenous antioxidants produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds. The serum levels of protein -SH in the body, can indicate antioxidant status. TNF-α is a polypeptide compound and it is an important member of the cytokine family, which plays a significant role in the regulation of the systemic inflammatory response.

The micro-rheological parameters, such as red blood cell deformability is influenced by the effect of ischemia-reperfusion. Red blood cell deformability is a pivotal ability of the cells to pass the capillary system which is required for sufficient tissue oxygenation. Deformability is determined by the internal viscosity of the cell, the membrane viscoelasticity, the surface-volume ratio and the morphology of erythrocytes. Mostly on the 1st – 4th postoperative days changes in red blood cell deformability are
related to the inflammatory reactions, hemodynamic alteration, induction of free-
radicals and mediators, acute phase reactions and changes in the coagulation state. In
the early hours of reperfusion metabolic and free radical alterations are more dominant.  
All these factors can further aggravate the postoperative complication of microvascular 
tissue transfer. Pathologically altered red blood cells show a reduction in their 
 deformability and may lead to capillary occlusion and decreased oxygen supply for the 
tissues. Most likely, most of these reactions (metabolic disturbance and induction of 
free radicals) are passed off by the 7th postoperative day; thereby we did not find any 
definitive difference on this day.

In the literature, there are controversies in the administration routes and doses of 
the TMZ. In our study 10 mg/kg dose was chosen and the drug was administered 
intraperitoneally, based on some previous studies where this dose was proved to be 
effective. The timing was also different in many studies. For example, Khan and 
colleagues published that TMZ was cardioprotective (via the activation of p38 
mitogen-activated protein kinase and Akt signaling pathway) when administered at the 
beginning of the reperfusion period. Elimadi et al. investigated the effect of TMZ on 
hepatic warm I/R injury, administered as an intramuscular injection with different doses 
(5 mg, 10 mg, 20 mg). They demonstrated that 10 mg/kg/day for 7 days before the 
induction of ischemia was the optimal dose, that gave the maximal protective effects at 
both the cellular and mitochondrial level. All these observed differences among the 
studies could be a consequence of different animal models, examined organs and I/R 
protocols. Further investigations are required to determine the optimal time and dose of 
administration of TMZ and to have more insight into clinical application.

In our study, we hypothesized that a single shot of TMZ will be preventive against 
I/R injury in epigastric skin flaps. Since in the previous studies the timing of the 
administration of TMZ was different, we investigated both pre- and postischemic TMZ 
treatment. Our data confirm the earlier findings, that TMZ has anti-inflammatory and 
anti-ischemic effects, independently of the timing. It could be a useful drug in the 
surgical practice to increase the survival time of the tissues, not just given before a 
planned ischemic period but also after an unexpected trauma where a reconstructive 
surgery is required.
4.5 CONCLUSION

The harmful effect of I/R can occur in the skin flaps without macroscopically visible changes (e.g.: tissue necrosis).

According to our results, TMZ is shown to be protective, against I/R injury and it is also suitable to decrease the inflammatory response. The administration of TMZ was effective independently of the timing: there is no unambiguous difference between the preischemic and postischemic TMZ administration. The beneficial effect of the postischemic administration can be especially important, because it can protect the tissues from ischemia-reperfusion injury, even after an unexpected ischemic insult.

Furthermore, TMZ is a clinically applied and nontoxic agent, which may increase the ischemic tolerance of the tissues and it is a promising drug to decrease the negative consequences of I/R in the surgical practice during free tissue transfer, replantation or even during revascularization procedures.
5 COMPARISON OF THE EFFECT OF TRIMETAZIDINE WITH ISCHEMIC PRE- AND POSTCONDITIONING IN REDUCING THE ISCHEMIA-REPERFUSION INJURY IN RAT SMALL INTESTINE

5.1 INTRODUCTION

Since 1959, when the first free jejunal flap was used by Seidenberg\textsuperscript{93} to reconstruct a cervical oesophageal defect, jejunal flaps have commonly been used for the reconstruction of oesophagus after cancer resections. Their main advantages are the followings: tubular structure similar to the oesophagus, available length, production of mucus, lack of functional gastrointestinal complication after removal, peristaltic activity similar to the pharyngoesophagus and the ease of preoperative preparation\textsuperscript{12,94}. Besides the oesophagus, the jejunal flap is also suitable for vaginal reconstruction (in the case of congenital absence of the vagina, sex assessment surgery, surgical resection of tumors), because it provides a durable, stable coverage and a tube passage for sexual intercourse. In these cases, jejunum transfer is a primary choice contrary to other intestinal transfers, particularly because it causes fewer defecation problems\textsuperscript{95,96}. However, the use of jejunal flaps is challenging, because of their sensitivity to ischemia and other technical details related to the operation. During the preparation of flaps, segments of the jejunum are removed and exposed to a period of warm ischemia until revascularisation. Although short ischemia times are associated with minimal damage, the injury from warm ischemia progresses even after revascularization. Ischemia-reperfusion injury (IRI) is a complex, multifactorial cascade of pathophysiological events, which include the depletion of oxygen to cells, low adenosine-triphosphate (ATP) stores, accumulation of toxic metabolites, reactive oxygen species, various cytokines, and other mediators, leading to further cellular dysfunctions. This structural injury might then endanger the functional outcome of the flap. Consequently, I/R induced tissue injuries are significant problems that might lead to different complications such as, segmental stenosis, fistula formation, peristalsis dysfunction, anastomosis leakage, and partial or complete flap failure\textsuperscript{97,98,99}. In spite of several suggested strategies/methods, so far effective, widely used method is not clinically available as a solution to this problem.

Ischemic preconditioning (IPreC) has been proved to produce resistance to the loss of blood supply and this method is able to improve the survival of the tissues,
subject to global ischemia. During IPreC brief period of ischemia followed by reperfusion is used, which increase the ischemic tolerance of the tissues against the detrimental effects of subsequent prolonged ischemia. First described by Murry and colleagues for myocardial tissue, since then, IPreC has been shown to increase the survival of a variety of flaps, subjected to ischemia. Beside the IPreC, ischemic postconditioning (IPostC) was also proved to be as effective as IPreC to improve the viability of the different tissues and organs/flaps after a prolonged ischemic insult. The main advantage of this method, that it is also able to reduce the degree of the damage even after an unexpected event.

Trimetazidine (TMZ) is a potent anti-ischemic drug and free radical scavenger, which besides the heart, has been protective in different organs against ischemia-reperfusion injury. Many studies have suggested that TMZ is able to reduce the intracellular acidosis, preserves the ATP production, limits the inflammatory reaction, the generation of reactive oxygen species and prevents the calcium overload of the cells, which can lead to further damage.

Our aim was to investigate the results of chemical preconditioning and postconditioning with trimetazidine, in a jejunal-flap model in the rat, compared its efficacy with ischemic preconditioning and ischemic postconditioning in reducing the oxidative stress and inflammation. Furthermore, the presence of any additive effects of simultaneous IPrec and TMZ; or IPostC and TMZ administration in improving the level of ischemic protection was also evaluated in this study.

5.2 MATERIALS AND METHODS

5.2.1 Animal model

Eighty male Wistar rats of the same age, weighing between 400 to 450 g, were used for this study. The rats were housed in separate cages, under standard conditions (temperature: 25±2°C, and air filtered room), with 12/12-hour light-dark regimen and were fed with standard rat chow and water ad libitum. Food was withdrawn 12 hours prior to the experiment. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation (number: ZOHU0104L 16).
5.2.2 Experimental protocol

In the first part of this experiment (A), we compared the effect of IPreC with TMZ preconditioning. The animals were divided randomly into five groups (10 rats in each group). The first group (Group 1) was the nonischemic control group. Although the control flaps did not undergo ischemic insult, laparotomy was performed. In the other groups (groups 2 through 5) ischemia was induced by placing a single microvascular clamp across the superior mesenteric artery (SMA). In the second group (I/R; Group 2) the SMA was clamped for 40 minutes, followed by 1 hour of reperfusion. The third (Preisch.TMZ+I/R; Group 3) group was the trimetazidine treated group. In this group, the TMZ was administered 30 minutes prior to the ischemic period. In the fourth group (IPreC+ I/R; Group 4) ischemic preconditioning was used: 2x5 min. (2 cycles of 5 minutes ischemia then 5 minutes of reperfusion) before the onset of ischemia. In the last group (TMZ, IPreC+ I/R; Group 5), animals received the TMZ 30 minutes prior to the ischemia and ischemic preconditioning was also performed, as mentioned above. (Fig.: 18)

![Diagram of experimental protocol](image)

*Fig. 18.*: Investigation groups for the comparison of TMZ and IPreC (first part of the study, A)
In the second part of the study (B), we compared the effect of IPostC with the TMZ postconditioning, and the additive effect of these two methods was also evaluated. Group 1 (nonischemic control); Group 2 (I/R) were the same as in the part A. However, the other groups were different: Group 3 (I/R + TMZ) was the TMZ treated group, but in this case, it was administered at the onset of reperfusion. In the fourth group (I/R+ IPostC) ischemic postconditioning was used: 3x30 sec (3 cycles of 30 secundum reperfusion, followed by 30-sec ischemia). In the last group (I/R+TMZ, IPostC) animals received TMZ at the onset of reperfusion and ischemic postconditioning was also performed, as mention above. (Fig.: 19)

![Diagram of investigation groups]

**Fig. 19.:** Investigation groups of the comparison of TMZ and IPostC. (second part of the study, B)

To standardize the study, all procedures were performed at similar time points in all groups. Animals, in the treated groups, received 10 mg/kg trimetazidine (trimetazidine-dihydrochloride, Sigma-Aldrich, St. Louis, Missouri, USA) intravenously (i.v), through the jugular vein. The drug was freshly solved into 0.9 % NaCl solution before the administration.
5.2.3 Surgical procedure

The rats have perioperatively anesthetized with an intraperitoneal (i.p) application of a mixture consisting of ketamine hydrochloride (5 mg/100 g) and diazepam (0.5 mg/100 g). The ratio was 1:1. During the operation, the animals were placed on a heated pad and ECG monitoring was also used. The carotid artery was catheterized (22 gauge) for blood pressure measurement. (Siemens Sirecust 1260, Düsseldorf, Germany). The skin of the abdomen was scrubbed with betadine and then laparotomy was performed. Then, the superior mesenteric artery was explored. Collaterals from the right colic and jejunal arteries were ligated as described by Megison et al\textsuperscript{107} (Fig. 20/A). Then according to the different groups, except in the control group, IRI was induced by placing a clip on the superior mesenteric artery for 40 minutes and trimetazidine, ischemic preconditioning/ischemic postconditioning or both were used. During the ischemic period the bowels were placed back into the abdominal cavity and the skin incision was temporarily closed.

After 40 minutes of ischemia, the microvascular clamp was released and the blood flow was confirmed by arterial pulsation, jejunal colour, and vascular patency tests were also performed to ensure that the blood flow is recovered successfully. The jejunal segment was reperfused for 60 minutes. At the end of the reperfusion period, approximately 10 cm segment of the jejunum (15 cm proximal to the ileocecal valve) was harvested. The resected jejunal segment was soaked in saline and then it was divided into 2 parts. One half was fixed in 4% neutral formaldehyde for histopathological examination and evaluation of apoptosis. The other half was instantly frozen in liquid nitrogen and stored at -80 °C for further biochemical examinations.

![Fig. 20.](image)

**Fig. 20.** On picture A, the schematic illustration of the model can be seen, described by Megison et al. Picture “B” and “C” are intraoperative pictures of the bowels before (B) and after (C) the clip application. Picture “B” represents a bowel with normal blood supply and normal color. Picture “C” shows bluish/pale discoloration of the bowel, because of the clamp.
5.2.4 Biochemical analysis

Jejunal segments and blood were taken for biochemical and histopathological examinations after 1 hour of reperfusion. The samples for the biochemical purpose were stored immediately at −80°C within individual containers. The tissue samples were stored in formalin, until the histopathological evaluations.

5.2.4.1 Oxidative stress parameters

MDA, GSH, SOD, and SH- levels were measured from the serum. MDA is a marker for the quantification of membrane lipid peroxidation. MDA levels were detected using a photometric method of Placer, Cushman, and Johnson. GSH and plasma SH levels were determined in anticoagulated whole blood by Ellman’s reagent, according to the method of Sedlak and Lindsay. Both indicate the antioxidative status of the body.

For the measurement of superoxide dismutase enzyme (SOD) activity from serum, the OxiSelect™ Superoxide Dismutase Activity Assay was used, following the manufacturer’s protocol. (Cell Biolabs Inc., STA-340)

5.2.4.2 Inflammatory cytokines

The inflammatory cytokine levels (TNF-α, IL-6) were studied by using the enzyme-binding immunosorbent assay (ELISA) method, following the manufacturer’s protocol (Assay Rat TNF-α ELISA kit, #AB46070; Assay Rat IL-6 ELISA kit, #AB119548, Abcam, Cambridge, UK).

5.2.5 Histopathological analysis

The histopathological study of the samples was carried out by the same pathologist. The tissue samples were fixed in 4% neutral buffered formaldehyde solution and embedded in paraffin. Three-micron-thick (Microtome: Thermo Scientific Microm Hm, 325) histological sections were cut, mounted on glass slides, stained with
hematoxylin-eosin (HE) and evaluated by light microscope to quantify intestinal mucosal injury, inflammation, necrosis or ulceration. Mucosal damage was graded from 0 to 5, based on the histologic injury scale, determined by Chiu et al.\(^{108}\). According to this:

- **grade 0:** Normal mucosal villi,
- **grade 1:** Development of subepithelial Gruenhagen’s space at the apex,
- **grade 2:** Moderate lifting of the epithelial layer at the apex of villi/Extension of subepithelial space;
- **grade 3:** Massive epithelial lifting down the side of villi;
- **grade 4:** Denuded villi and dilated capillaries/increased cellularity of lamina propria;
- **grade 5:** Disintegration of lamina propria/ Hemorrhage and ulceration.

For the detection of apoptotic cells, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was also performed.

### 5.2.6 Statistical analysis

For statistical evaluation, one-way analysis of variance (ANOVA) was used, followed by adequate post hoc tests (Dunnett’s, Sidak) for multiple comparisons. All data are represented as the mean±SEM. The difference was considered statistically significant when the \( p \) value was less than 0.05.
5.3 RESULTS

5.3.1 Changes of biochemical parameters in blood

5.3.1.1 Biochemical results of the comparison of TMZ and IPreC treatment
Reduced Glutathione (GSH) and Malondialdehyde (MDA)

The GSH levels were significantly lower in all groups compared to the control group (I/R: 883±16,15 p<0,0001; TMZ before isch: 1179±35,74 p=0,0001; IPreC: 1156±33,18 p<0,0001; TMZ+IPreC: 1271±34,71 p=0,0436 vs. Control: 1389±35,89). Compared to the I/R group, significantly higher values were measured in all treated groups (p<0,0001 in all cases). These findings support the antioxidant effect of the applied methods.

The MDA levels were considerably higher in all groups compared to the control group (I/R: 61,83±1,6 p<0,0001; TMZ before isch.: 40,25±0,98 p=00025; IPreC: 45,86±1,003 p<0,0001; TMZ+IPreC: 40,30±0,83 p=0,0023 vs. Control: 34,41±1,1). The MDA level was the highest in the I/R group. Comparing the different treated groups with each other, it can be seen, that in those groups where TMZ was administered (only itself or together with IPreC), the MDA levels were significantly lower than in IPreC group (IPreC vs. TMZ before isch.: p=0,0062; IPreC vs. TMZ+IPreC: p=0,0068). This suggests that the TMZ has a stronger effect against the lipidperoxidation. (Fig.21)

![Fig.21](image)

*Fig.21.* Plasma concentration of reduced glutathione and malondialdehyde in the investigated groups. GSH serves as a marker of the antioxidant status. MDA serves as a marker of the lipid peroxidation. *: p <0,05 vs. control; #: versus I/R p<0,05; +: p <0,05 among the signed groups; error bars: SEM.
**Sulphhydryl groups (SH-) and superoxide dismutase enzyme activity (SOD)**

SH-levels were also reduced in all groups comparing to the control (I/R: 39.45±0.95 p<0.0001; TMZ before isch.: 48.07±1.81 p<0.0001; IPreC: 41.51±1.08 p<0.0001; TMZ+IPreC: 46.54±1.07 vs. Control: 66.83±1.43). In those groups where TMZ was administered the SH-levels were significantly higher than in I/R group (TMZ before isch vs I/R: p=0.0002.; TMZ+IPreC vs. I/R: p=0.0023). Furthermore, in the IPreC group, the SH-levels were considerably decreased compared to that group, which received the TMZ before the ischemia (p=0.0055).

The SOD enzyme activity was also increased in all treated groups comparing to the I/R group, although the IPreC group was less elevated than the TMZ treated groups. The best result was in that group, where the TMZ and IPreC were also applied. *(Fig. 22)*

Both findings support our previous results, that TMZ has stronger antioxidant property than the IPreC.

Fig. 22.: Concentrations of SH- groups, and SOD enzyme activity in the plasma. The levels of SH- and SOD refer to the antioxidant status. Error bars: SEM.
**Inflammatory cytokines: TNF-alfa, IL-6**

On the first diagram, the results of TNF-alfa measurement can be seen. The TNF-alfa levels were elevated in all groups compared to the control (I/R: 27.49±0.65; TMZ before isch.: 20.33±0.55; IPreC: 21.05±0.54; TMZ+IPreC: 18.36±0.60 p<0.0001 in all cases vs. Control: 13.57±0.85). We measured significantly decreased values in all treated groups compared to the I/R group (p<0.0001 in all cases). Among the treated groups considerable changes were seen only between the IPreC and TMZ+IPreC groups (p=0.0387).

The results of the IL-6 were similar to the TNF-alfa level, except that the last group (combined treated) did not show significant elevation compared to the control (I/R: 132.5±3.37 p<0.0001; TMZ before isch.: 92.86±0.26 p=0.0063; IPreC: 95.62±0.42 p=0.0002; TMZ+IPreC: 90.32±0.79 p=0.0971 vs. Control: 85.28±0.47). Considerable differences were not seen among the treated groups, but compared to the I/R group, all treatment caused significant drop in the IL-6 levels.

All kind of treatment has anti-inflammatory properties to some extent, but the combination of TMZ and IPreC seems to be the most effective method. (Fig. 23.)

![Diagram](image)

**Fig.: 23.** TNF-alpha and IL-6 concentrations show the grade of the inflammatory response in the investigated groups. *: p<0.05 vs. control; #:/+: p<0.05 among the signed groups; error bars: SEM
5.3.1.2 Biochemical results of the comparison of TMZ and IPostC treatment
Reduced Glutathion (GSH) and Malondialdehyde (MDA)

The GSH levels were significantly lower in all groups, except the last one, compared, to the control group (I/R: 882.6±16.15; TMZ before rep.: 1112±33.96; IPostC: 1047±26.16 p<0.0001 in all cases; TMZ+IPostC: 1351±54.54 p=0.7031 vs Control: 1400±30.8). Compared to the I/R group, significantly higher values were measured in all treated groups (I/R vs. TMZ before rep.: p=0.0002; vs. IPostC: p=0.01; vs. TMZ+IPostC: p<0.0001). The best results were found in the last group, which received also TMZ and IPostC.

The MDA levels were considerably higher in all groups, except the last, compared to the control group (I/R: 61.83±1.6 p<0.0001; TMZ before rep.: 41.36±0.95 p=0.0003; IPostC: 42.55±0.68 p<0.0001; TMZ+IPostC: 35.73±1.1 p=0.8207 vs. Control: 34.41±1.076). The MDA level was the highest in the I/R group. Comparing the different treated groups with each other, the best result was found in the combined treated group, where the MDA levels were significantly lower than in the other two treated groups (vs. TMZ before rep.: p=0.0055; vs. IPostC: p=0.0005). (Fig. 24)

These findings support the antioxidant effect of the applied methods, and suggest that TMZ can improve the effect of IPostC, and the best results occur when the two methods are combined with each other.

![Fig. 24: Plasma concentration of reduced glutathione and malondialdehyde in the investigated groups. GSH serves as a marker of the antioxidant status. MDA serves as a marker of the lipid peroxidation. *: p <0.05 vs. control; #: p <0.05 among the signed groups; error bars: SEM.](image-url)
**Sulphhydryl groups (SH-) and superoxide dismutase enzyme activity (SOD)**

SH-levels were also reduced in all groups comparing to the control (I/R: 39,45±0,95; TMZ before rep.: 50,25±2,03; IPostC: 39,82±0,95; TMZ+IPostC: 48,37±1,98 p<0,0001 in all cases). In those groups where TMZ was administered (TMZ before rep.: p<0,0001; TMZ+IPostC p=0,001 vs. I/R) the SH- levels were significantly higher than in I/R group. Furthermore, in the IPostC group, the SH- levels were considerably decreased compared to those groups, which received the TMZ (TMZ before rep.: p=0,0001 and TMZ+IPostC: p=0,0018).

The SOD enzyme activity was significantly lower in all groups compared to the control (I/R: 281,5±20,24; TMZ before rep.: 528,6±5,33; IPostC: 377±11,39 p<0,0001 in all cases; TMZ+IPostC: 612,9±1,75 p=0,0009 vs. Control: 663,1±7,75), and significantly higher in all treated groups comparing to the I/R group (p<0,0001 in all groups), although the values of IPreC group was less elevated than the TMZ treated groups (IPostC vs. TMZ before rep./TMZ+IPostC p<0,0001). The best result was in that group, where the TMZ and IPostC were also applied (TMZ+IPostC vs. TMZ before rep.: p<0,0001). *(Fig. 25.)*

Both findings support that TMZ itself has stronger antioxidant property than the IPostC, however, the combination of TMZ and IPostC provided the best results.

![Fig.: Concentrations of SH- groups, and SOD enzyme activity in the plasma. The levels of SH- and SOD refer to the antioxidant status. #: versus control: p<0,05 versus I/R p<0,05 among the signed groups; Error bars: SEM.](image-url)
**Inflammatory cytokines: TNF-alfa, IL-6**

On the first diagram, the results of TNF-alfa measurement can be seen. The TNF-alfa levels were elevated in all groups compared to the control (I/R: 27,49±0,65 p<0,0001; TMZ before rep.: 17,72±0,97 p=0,0028; IPostC: 21,46±0,82 p<0,0001; TMZ/IPostC: 17,86±0,52 p=0,002 vs. Control: 13,57±0,855). We measured significantly decreased values in all treated groups compared to the I/R group (p<0,0001 in all cases). Considerable changes were seen among TMZ treated groups (TMZ before rep.: p=0,0116 and TMZ/IPostC p=0,0162 vs. IPostC.) and IPostC group.

The results of the IL-6 were similar to the TNF-alfa level, regarding the control (I/R: 132,5±3,37; TMZ before rep.: 97,81±0,51; IPostC: 106,5±0,39; TMZ/IPostC: 100,2±1,17 p<0,0001 in all groups) and I/R groups (p<0,0001 in all cases). Among the treated groups, a significant difference was measured only between the TMZ before rep. group and the IPostC group (p=0,0038).

All kind of treatment has anti-inflammatory properties to some extent, however, the TMZ itself and, the combination of TMZ and IPostC seems to be more effective methods, than the IPostC itself. (Fig. 26)

![Fig. 26:](image)

Fig. 26.: TNF-alpha and IL-6 concentrations show the grade of the inflammatory response in the investigated groups. *: p<0,05 vs. control; #/+: p<0,05 among the signed groups; error bars: SEM
Histopathological results

5.3.1.3 Hematoxylin-eosin staining

For the determination of intestinal mucosal injury: inflammation, necrosis or ulceration, HE-staining was performed. For the histopathological evaluation, we used the Chiu score which distinguishes 6 grades from each other, from grade 0-grade 5. Grade 0 represents the normal mucosal villi, and in grade 5 disintegration of lamina propria, hemorrhage and ulceration can occur. In the control group, we can see normal mucosal villi, without any signs of the mucosal injury (Fig.27.). In our study, the nontreated ischemic-reperfusion group (I/R) was between grade 4 and 5 with denuded villi and disintegration of lamina propria (Fig.28.). Administration of TMZ before ischemia was more effective than the administration of the drug before the reperfusion period (Fig.29-30.). According to our histopathological findings IPreC itself is more effective than the IPostC, and its effectiveness is similar to TMZ when it was given before the reperfusion period (Fig.31-32.). Based on our histopathological results, the best outcome was in the last group where both TMZ and IPostC were used. Furthermore, the additive effect of TMZ and IPostC was stronger than TMZ and IPreC (Fig.33-34.).

Fig. 27.: In the control group, the structure is kept, normal mucosal villi can be seen. (HE, 5x)
(Grade 0)

Fig. 28.: In the I/R group denuded villi and disintegration of lamina propria were found. (HE, 5x)
(Grade 4-5)
Fig. 29.: Development of Gruenhagen’s space (arrows) and moderate lifting of the epithelial layer at the apex of villi can be seen in the group of TMZ before ischemia. (HE, 7.3x)
(Grade 1-2)

Fig. 30.: In the group of TMZ before reperfusion, moderate lifting of the epithelial layer at the apex of villi is demonstrated. (HE, 7.3x)
(Grade 2)

Fig. 31.: In IPreC group extension of subepithelial space is presented. (HE, 7.3x)
(Grade 2)

Fig. 32.: In IPostC group epithelial lifting down the side of the villi, extension of subepithelial space and dilated capillaries were found. (HE, 7.3x)
(Grade 3)
Fig. 33.: In TMZ+IPreC group development of subepithelial Gruenhagen’s space (arrows) at the apex and moderate lifting of the epithelial layer at the apex of villi can be seen. (HE, 7,3x) (Grade 1-2)

Fig. 34.: In TMZ+IPostC group only development of subepithelial Gruenhagen’s space (arrows) at the apex is presented (HE, 7,3x) (Grade 1)
5.3.2.2. TUNEL-staining

In the control group, positive cells (staining brown) were barely seen in the crypts. However, compared with the control group, in the I/R group, the amounts of the positive cells were significantly higher. Within the treated groups, we can see a tendency according to the treatments: the worse results were shown in the IPostC group, followed by the IPreC, TMZ before isch., TMZ before rep.; TMZ+IPreC and TMZ + IPostC groups. The same pathologist, who performed the TUNEL-staining valued the results according to the number of the positive cells in 8 crypts (from a representative area). The similar treated groups (e.g: IPreC-IPostC; TMZ before isch-TMZ before rep.; TMZ+IPreC- TMZ+IPostC) did not show any considerable differences. Nevertheless, the drop in the number of positive cells was significant between the combined treated groups (TMZ+IPreC; TMZ+IPostC) and the ischemic pre/ or postconditioned groups. Compare the number of the positive cells in the treated groups to the I/R group, a considerable decrease was detected in all cases, however, the most significant reduction was found in the combined treated groups.

![Fig.35. TUNEL-staining, magnification 10x: TUNEL staining demonstrates the apoptotic nuclei. There is a significant difference in the number of apoptotic cells between the control (first picture) and the I/R group (second picture): a high number of TUNEL-positive nuclei were detected in the crypts after I/R.](image-url)
Fig. 36.: TUNEL-staining, magnification 10x: Comparison of the effect of the different treatments. The best outcome was found in the combined treated groups (TMZ+IPreC; TMZ+IPostC), and the most apoptotic areas were demonstrated in the ischemic pre- and postconditioned groups. These findings also support that the TMZ has a stronger protective effect than the IPreC/IPostC against apoptosis, induced by I/R.
5.4 DISCUSSION

The jejunum is one of the most frequently used free flap in the head and neck region for pharynx and cervical oesophagus reconstruction, but it is also a suitable flap for the reconstruction of the vagina. However, the jejunum is one of the most sensitive tissues to ischemia-reperfusion injury in the body, so the success of intestinal transplantation is highly influenced by the susceptibility of the small bowel to IRI, which inescapably affects the graft. This property of jejunal flaps is also an important stress factor for the surgeons during the operation (vessel anastomosis) and in the postoperative phase. Although the success rates are mainly high (around 90-95 percent), ischemic injury has been implicated as one of the most important etiologic factors in the occurrence of postoperative complications like fistula formation and stenosis.

During the free jejunal flap transfer, the ischemic period is unavoidable, which together with the subsequent reperfusion, can lead to serious mucosal injury. Structural damage can be established microscopically, already several minutes after the onset of the ischemic insult\textsuperscript{109}. The underlying mechanism of the rapid destruction is not unambiguous yet, although acid provoked disruption of lysosomal membranes, depletion of cellular energy stores and accumulation of toxic metabolites might be involved\textsuperscript{110}. The sudden oxygen influx during reperfusion of the ischemic tissues aggravates the damage via the generation of oxygen free radicals, apoptosis and the production of inflammatory mediators. Inflammatory response involves the increased expression of endothelial cell adhesion molecules, complement activation, endothelial barrier dysfunction, increased recruitment of leucocytes and macrophages, nuclear transcription factor κB activation and consequent overexpression of pro-inflammatory cytokines, including TNF-alfa, IL-1 β, IL-6, IL-8. Inflammatory changes of the small bowel mucosa, caused by IRI, can result in increased enterocyte apoptosis, villous ulceration, epithelial sloughing and leukocyte and platelet adhesion to intestinal microcirculation\textsuperscript{111,112,113,114,115}.

In the present study, we explored the effect of the Trimetazidine (TMZ) treatment, compared to the ischemic preconditioning (IPreC) or ischemic postconditioning (IPostC), on immunological and morphological changes, induced in the small bowel mucosa by I/R.

There is a significant amount of data from animal studies indicating the safety and efficacy of ischemic preconditioning and ischemic postconditioning. The common of
the two latter methods is the usage of short sublethal cycles of I/R prior, or right after the prolonged ischemic insult, providing protection against IRI. There is a lot of studies, which proved the effectiveness and the ability of the two methods to increase the ischemic tolerance of different organs and tissues, like the myocardium, brain, liver, kidney, jejunum, skin and muscle flaps \(^{116,117,118,119}\).

Ischemic preconditioning was first described by Murry \(^{100}\) et al., and since then this topic is still in the public eye. Either local (applied on a target organ) and remote IPreC (applied on remote organs/tissues) are well known. The molecular mechanisms for this effect include modification in adenosine triphosphate metabolism \(^{120}\), decrease of neutrophil-mediated reperfusion injury, activation of K\(_{ATP}\) channels and therefore prevention of intracellular Ca\(^{2+}\)-overload. Decreased glycogen breakdown is also an important part, hereby IPreC preserves energy substrate. Protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-kinase), Akt, nitric oxide synthase (NOS), guanylyl cyclase, protein kinase G (PKG) and other molecular intermediates are also involved \(^{121}\).

First Zhao et al. \(^{122}\) described that the ischemic postconditioning is similar to the ischemic preconditioning, and it is able to reduce the degree of myocardial IRI equally. The mechanism in the background of ischemic postconditioning is still remaining unclear, probably the signaling pathway is similar between the IPreC and IPostC. It has been proved that IPostC is able to protect the mitochondrial integrity through the regulation of mitochondrial permeability transition pores (mPTPs), it can reduce the sensitivity to the increased intracellular calcium overload and the restoration of nitric oxide-mediated vasorelaxation is also an important effect of the method \(^{123,124}\).

Trimetazidine (TMZ), \([1-(2,3,4 \text{trimetoxibenzyl})-piperazine \text{dihydrochloride}]\), is an effective, well-tolerated drug mainly used in angina pectoris. The favourable effect of TMZ treatment in patients with ischemic heart disease and heart failure is well documented and the anti-ischemic, anti-inflammatory effects of the drug are reported on other ischemic organs as well. Among the possible mechanisms, that could be responsible for these effects were suggested the prevention of the intracellular decrease of ATP levels and the intracellular acidosis, reduction in ROS toxicity, decrease in the inflammatory reaction, reduction in the utilization of fatty acids, prevention of Ca\(^{2+}\)-induced mPTP opening \(^{125,126,127,128}\). It is also proved that TMZ has no alteration in hemodynamic parameters and besides the fact that TMZ can worsen the symptoms of Parkinson’s disease and gait disorders, only episodes of a headache were mentioned as a side effect \(^{36,129}\).
For the determination of the tissue damage and the level of the oxidative stress, the following parameters were evaluated: MDA, GSH, SH, SOD. To gain information about the inflammatory response IL-6 and TNF-α were measured. MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. GSH, SH, and SOD are the members of the antioxidant systems, therefore an increase in the number of reactive oxygen species leads to the depletion of these parameters. That explains our results, namely the highest MDA levels and the lowest GSH, SH levels and the lowest SOD enzyme activity were measured in the I/R group. In all treated groups, especially where TMZ was used, a reduced oxidative stress with a smaller elevation of MDA (p<0.05), and a less depletion of antioxidant systems (SOD, GSH, SH) were detected.

The current study also showed that I/R induced inflammatory response, which was demonstrated by a significant elevation in the proinflammatory cytokines levels including TNF-α, IL-6. Our different treatments were also able to decrease the inflammation.

During our histopathological examinations, we used a scoring method, created by Chiu et al\textsuperscript{10}, which describes the morphological changes and mucosal damages, associated with IRI in the intestine. According to them the Grade 0 is the normal intestine, Grade 1 means the development of subepithelial Gruenhagen’s spaces at the apex. These spaces develop as enterocytes lift off the basement membrane. As the extent of IRI increases moderate lifting of the epithelial layer at the apex of villi or extension of the subepithelial space can be noticed (Grade 2). At an advanced stage, massive epithelial lifting occurs down the side of the villi (Grade 3) or denuded villi, dilated capillaries and increased cellularity of lamina propria can be seen (Grade 4). The signs of the final stage are the followings: the disintegration of lamina propria, hemorrhage, and ulceration (Grade 5). In our study Grade 0- Grade 4 appeared. After the hematoxylin-eosin staining, TUNEL-staining was also applied in all groups. This method is able to establish the level of apoptosis. In our case, the most involved area was in the I/R group, where the number of apoptotic cells was the highest. The best results were found in the control group, however, the results of TMZ+IPreC and TMZ+IPostC were nearly as good as in the control.

Earlier Tetik et al\textsuperscript{85} also investigated the cytoprotective effect of TMZ in the rats against the intestinal ischemia-reperfusion injury. They administered 3 mg/kg TMZ intravenously 10 minutes before the induction of ischemia. The ischemic period was 60
minutes which was followed by 120 minutes of reperfusion. In this study only the measurement of MDA, myeloperoxidase levels were evaluated and HE-staining was assessed. According to their results, TMZ pretreatment attenuated but did not prevent histological damage from I/R by inhibiting lipid peroxidation and neutrophil infiltration in the mucosal tissue. In contrast with this study, we applied only 40 minutes of ischemia, followed by 60 minutes of reperfusion and the dose of TMZ was 10 mg/kg, based on previous studies. We chose the 40 minutes of ischemia because this was described as a critical ischemia time in the small bowel in a transplantation model, even if the intestinal damage occurs already within 15 minutes of ischemia. Interestingly, during the reperfusion, the small bowel has the ability to start a self-repairing mechanism, so the previous mucosal damage may disappear, if the reperfusion period is long enough. Therefore, we preferred to use only 60 minutes of reperfusion in our study.

In another study Yalcin et al.\textsuperscript{130} investigated the effect of trimetazidine on burn-induced intestinal mucosal injury and kidney damage in rats. They used 3mg/kg TMZ, and they found that TMZ decreased MPO levels, but there was no effect on GSH/GSSG and MDA levels. The explanation of the different results in MDA and GSH levels between our study and theirs, can be caused by the different dose of the drug, and also the severity of the established ischemia. In our experiment, the length of the ischemic pre- and postconditioning was established, according to earlier studies, where the most optimal time for ischemic preconditioning in bowel was 2 x 5 minutes and the best option for ischemic postconditioning was 3x 30 sec\textsuperscript{131,132}.

In this study, our aim was to compare the effect of TMZ with ischemic pre- and postconditioning, and to evaluate the effect if the TMZ and IPreC/IpostC are used in a combination.

Based on our study, the treatment with TMZ is promising, and it could be a useful drug during free jejunal flap surgeries or in any case, where intestinal I/R injury occurs. Undoubtedly, further studies are required to find the optimal usage of the drug in human surgeries.
5.5 CONCLUSION

All types of conditioning alone, or in combination, decreased the oxidative stress and the inflammation and improved histopathological appearance. However, according to our results, the pharmacological preconditioning/postconditioning with TMZ alone, seemed to be more effective in the jejunum, than the ischemic pre-/or postconditioning. Furthermore, TMZ was able to increase the efficacy of both above-mentioned methods. As a conclusion, TMZ is a promising drug to increase the ischemic tolerance of the tissues, and it may have an important role not just in cardiology but also in the surgical field.
6 NOVEL FINDINGS

In our first study, we investigated the role of BFSP1 protein, in human breast cancers. Based on our results we can confirm the followings:

a) BFSP1 is presented not only in the eye lens but also in ex vivo human breast cancer.

b) The same variants of BFSP1 protein occur in the tumour samples and also in the serum.

c) There is a difference in the contents of BFSP1 according to that, the tissue samples are from a tumour or from a tumour-free area.

d) BFSP1 protein may become a new diagnostic tool for breast cancers (histopathological type: carcinoma ductale infiltrans) and can be useful during the diagnostic period and also in the follow-up phase.

In the second and third study, we demonstrated the effect of trimetazidin (TMZ) against ischemia-reperfusion injury in skin flaps and, in the jejunum. According to these studies we can conclude that:

a) The harmful effect of ischemia-reperfusion can occur in the flaps without macroscopically visible changes

b) TMZ is able to decrease the level of oxidative stress parameters (MDA, GSH, SH-, SOD) and also inflammatory response (TNF-alfa, IL-6, histopathology).

c) TMZ can reduce the number of apoptotic cells.

d) There is no unambiguous difference between the preischemic and postischemic TMZ administration, however, both of them were effective methods to increase the ischemic tolerance of the distal parts of skin flaps.

e) The same dose of TMZ (10 mg/kg), we used in skin flaps was also effective in the jejunum.

f) TMZ alone is more effective (independently the time of the administration: even it is given before ischemia or at the onset of reperfusion period) than the ischemic pre (IPreC)- or postconditioning (IPostC) itself.

g) TMZ is able to improve the anti-ischemic effect of ischemic pre-and postconditioning. This effect is stronger, when the TMZ is applied together with IPostC.
7 ACKNOWLEDGEMENT

I would like to take this opportunity to express my thanks for the overwhelming support I have received from my supervisors Gábor Jancsó MD, and Ildikó Takács MD. in completing this work.

I would also like to acknowledge the help and assistance of Tibor Nagy MD, Péter Hardi MD, Dr. János Lantos and of all the staff (Ágnes Pázmándy, Adrienn Jakabovics, Erika Átol, Gábor Mák, Nikolett Buza) at the Department of Surgical Research and Technique of Pécs University to carrying out the investigations and giving me the inward support over the years.

I am grateful for Gábor Pavlovics MD, and György Tizedes MD. for the professional support what they provided me in the field of reconstructive surgeries.

I am thankful for Antal Tapodi MD, Endre Kálmán MD and Gyula Kovács MD, for the common work and for the indispensable help with the study of BFSP1, in the Department of Biochemistry and Medical Chemistry, Pathology Department and at the Surgery Clinic.

I would like to express my thanks to Péter Kenyeres MD., and to Dóra Praksch MD., from the Hemorheological Laboratory of 1st Department of Medicine.

Finally, I would like to give thanks to my parents and my family for their patience and love, and I appreciate that they supported me all the time.

This work could not be prepared without the financial support which was provided by „EFOP 3.6.1-16-2016-00004”.
8 LIST OF PUBLICATIONS AND PRESENTATIONS

8.1 Scientific publications/presentations related to the topic of this PhD


- L. Petrovics, I. Takacs, G. Jancso, T. Nagy, P. Hardi, F. Németh, Z. Trojnár, L. Bognar, P. Varga, B. Gasz, G. Pavlovics: Comparison the effect of chemical preconditioning with ischemic preconditioning, in reducing the ischemia-reperfusion injury in rat small intestine; 53rd Congress of the European Society for Surgical Research (ESSR 2018), Madrid, Spain; 05.30-06.02.2018. (Poster section 1. price)


- Petrovics Laura, Nagy Tibor, Hardi Péter, Németh Franciska, Trojnár Zoltán, Takács Ildikó, Pavlovics Gábor, Jancsó Gábor: Iszkémia-reperfúziós károsodások csökkentése ismert anti-iszkémiai szerrel, patkány bőrlebenyre,
A Magyar Sebész Társaság Kísérletes Sebészeti Szekciójának XXIV. Kongresszusa, Herceghalom, 2017. szeptember.29

- **L. Petrovics**, F. Németh, Z. Trojnár, T. Nagy, P. Hardi, G. Pavlovics, I. Takács, G. Jancsó Ischaemia-Reperfusion Injury Is Reduced by an Anti-Ischaemic Agent in Skin Flaps, **52nd Congress of the European Society for Surgical Research (ESSR 2017), Amsterdam, The Netherlands; June 2017**

Eur Surg Res 2017;58(suppl 2):1–69 51 DOI: 10.1159/000479831 Published online: September 21, 2017


- Németh F., Trojnár Z, **Petrovics L.**: Trimetazidin szerepe az iszkémia-reperfúziós károsodások csökkentésében, patkány bőrlebenyek esetén, Grastyán Konferencia, Pécs, 2017.03.29-31

### 8.2 Other scientific presentations


• Sárvári K., Petrovics L., Veres Gy. T.: Role of pre-and postconditioning to avoid noxious oxidative stress induced by pneumoperitoneum, Ninth YES-Young European Scientist- Meeting, September 2014


• Katalin Sárvári, Laura Petrovics, Tünde Gyöngyvér Veres; A prekondícionálás szerepe a pneumoperitoneum okozta káros oxidatív hatások kivédésében/ Role of preconditioning to avoid noxious oxidative stress induced by pneumoperitoneum, HMAA (Balatonfüred), 2014. augusztus


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The effect of trimetazidine in reducing the ischemia-reperfusion injury in rat epigastric skin flaps

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Abstract.
BACKGROUND: Ischemia-reperfusion injury may lead to insufficient microcirculation and results in partial flap loss during the free flap surgeries.
OBJECTIVE: This study aimed to investigate the effect of trimetazidine (TMZ) on oxidative stress, inflammation and histopathological changes, using the epigastric skin flap model in rats.
METHODS: 40 male Wistar rats were used, that were divided into four groups. Control group, non-treated ischemic (I/R)-group and two trimetazidine treated groups (preischemically, postischemically) were established. To create ischemia in the skin flap, the superficial epigastric vessels were clamped for six hours, followed by twenty-four hours of reperfusion. Blood samples and biopsies from skin flaps were collected at the end of the reperfusion period. The inflammatory response, the degree of oxidative stress (by measuring the plasma level of malondialdehyde (MDA), reduced glutathione (GSH); sulfhydryl (–SH) groups) and histopathological changes were evaluated.
RESULTS: Inflammatory response, and oxidative stress were significantly attenuated in the trimetazidine treated groups, compared to the non-treated ischemic group. Histopathological findings were also correlated with the biochemical results.
CONCLUSION: In our study trimetazidine could reduce the ischaemia-reperfusion injury, even after an unexpected ischemic period, so it is a promising drug during free tissue transfer, replantation or during revascularization procedures in the future.

Keywords: Ischaemia-reperfusion injury, free flaps, reconstruction, trimetazidine, inflammation, oxidative stress

1. Introduction

Ischemia-reperfusion (I/R) injury can cause considerable problems in various fields of the surgery, like in reconstructive plastic surgery, vascular surgery, traumatology or cardiac surgery. Ischemia-reperfusion injury is a cascade of pathophysiological events, that can occur after the reperfusion of the tissues, exposed to prolonged ischemia and results in tissue damage [1, 2]. Unfortunately, this condition is unavoidable during free flap surgery or during replantation. Free tissue transfer has become a routine procedure to cure tissue defects after oncological ablative surgery or trauma. In the last decade, the technique of the free flap surgeries improved a lot and it has reached the 90–95\% success rate. Although, the success rates of these surgeries are high, there are still some cases, where the insufficient microcirculation, caused by I/R injury, leads to partial flap loss and results in the reoperation of the patient. In addition, the flap/limb can become irremediable because its poor circulation, and it may make
the reconstruction more difficult or impossible [3–7]. For these reasons the detection of biochemical changes and microcirculatory disorders in flaps during I/R, are of high importance [8, 9].

Even though many drugs and methods have shown promising results experimentally, there is not an existing consensus treatment in the clinical practice, because of their unfavourable systemic side effects, excess toxicity, limited efficacy, invasive administration or because of the time-consuming technique [10–15].

Trimetazidine (TMZ, water soluble form: trimetazidine-dihydrochloride) is a widely used anti-anginal drug worldwide. It is a potent anti-ischemic agent and a free radical scavenger. It has been used in many studies to protect different organs (myocardium, the intestine, liver, and kidney) from the ischemia-reperfusion injury. Numerous evidence exists, which shows that the reperfusion injury could be decreased by TMZ-preconditioning in animals. It was found that TMZ conserves ATP production, maintains cellular homeostasis and reduces the intracellular acidosis [16]. Moreover, it decreases the oxidative damage to the mitochondria and protects the organ from tissue damage, induced by I/R injury [17, 18]. Furthermore, Devynck et al. investigated the effect of TMZ on membrane in human platelets and found that TMZ reduced cAMP content and aggregation responses to collagen and ADP [17]. TMZ is accepted as an agent without any hemodynamic activities, and only minor side effects (episodes of a headache) were mentioned in a few cases [19].

We hypothesised that a single shot of TMZ will be protective against I/R injury. This study aimed to investigate the effect of trimetazidine on oxidative stress, inflammation, and histopathological alterations (before visible changes (e.g. tissue necrosis) occur), using the epigastric skin flap model. To determine the efficacy of TMZ, levels of blood malondialdehyde (MDA), reduced glutathione (GSH), and plasma thiol groups (SH-) and tissue TNF-alpha were measured, histopathology and immunohistochemistry were performed.

2. Material and methods

2.1. Animal model

Forty male Wistar rats of the same age, weighing between 350 to 400 g, were used for this study. The rats were housed in separate cages, under standard conditions (temperature: 25 ± 2°C, and air filtered room), with 12/12-hour light-dark regimen and were fed with standard rat chow, and water ad libitum. Food was withdrawn 12 hours prior to experiment. The study protocol was approved by the National Scientific Ethical Committee on Animal Experimentation (number: ZOHU0104L 16).

2.2. Experimental protocol

The animals were divided randomly into four groups (10 rats in each group). The first group was the non-ischemic control group. Although the control flaps did not undergo ischemic insult, flap harvest produced some temporary ischemia. In the other groups (groups 2 through 4) ischemia was induced by placing a single microvascular clamp across the epigastric superficial artery and vein. In the second group (I/R) the superficial epigastric vessels were clamped for 6 hours, followed by 24 hours of reperfusion. The third (Preisch.TMZ+I/R) and fourth (I/R+Postisch.TMZ) groups were the trimetazidine treated groups. In the third group, the TMZ was administered 30 minutes prior to the ischemic period. In the last group, animals received the drug at the onset of the reperfusion (Fig. 1). To standardize the study, all procedures were performed at similar time points in all groups. Animals, in the treated groups, received 10 mg/kg trimetazidine (trimetazidine-dihydrochloride, Sigma-Aldrich, St. Louis, Missouri, USA) intraperitoneally (i.p) depending on the groups, 30 minutes prior to ischemia
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Fig. 1. Investigation groups: I: ischemia, R: reperfusion, TMZ: trimetazidine.

(Preisch.TMZ+I/R) or at the onset of the reperfusion (I/R+Postisch.TMZ). The drug was freshly solved into 0.9 % NaCl solution before the administration.

2.3. Surgical procedure

The rats were perioperatively anesthetized with an intraperitoneal (i.p) application of a mixture consisting of ketamine hydrochloride (5 mg/100 g) and diazepam (0.5 mg/100 g). The ratio was 1:1. The skin of the abdomen was depilated using an animal depilatory agent. During the operation, the animals were placed on a heated pad and ECG monitoring was also used. The carotid artery was catheterized (22 gauge) for blood pressure measurement. (Siemens Sirecust 1260, Düsseldorf, Germany). The skin of the abdomen was scrubbed with betadine and then 3 × 6 cm flap was created on both sides of the abdomen. In our study, the epigastric flap was chosen, because it simulates microsurgical free tissue transfer closely. This model was first described in 1967 by Strauch and Murray and has been widely used in various experimental animal researches on I/R injury and skin flap survival [20–23]. The flaps include the area within the boundaries of costal arch as an upper limit, the inguinal ligament as a lower limit and both axillary lines as lateral borders. The medial borders were on both sides of the midline structures (the xiphoid and pubis). The vascular supply of the flap is provided by the medial and lateral branches of the superficial epigastric artery and accompanying veins, based on the superficial epigastric vascular pedicle. After 6 hours of ischemia, the microvascular clamp was released and the blood flow was confirmed by arterial pulsation, flap colour, and the vascular patency test was also performed to ensure that the blood flow is recovered successfully. Flaps, where we could not detect any flow, were not included in this study. After checking the blood flow, the skin was sutured back to its original place with interrupted stitches (5–0, Prolene (Ethicon), 30 stitches on both flaps). After the operation, the animals got a collar neck to prevent the automutilation. On the next day, before the sampling, animals were re-anesthetized.

Skin samples (3 × 1 cm) were taken from the most distal end of the flaps, after 24 hours of reperfusion, for biochemical examination. The samples were stored immediately at –80°C within individual
containers. MDA, GSH, SH levels were measured from these skin samples. MDA is a marker for the quantification of membrane lipid peroxidation. MDA levels were detected using a photometric method of Placer, Cushman and Johnson [24]. GSH and plasma SH levels were determined in anticoagulated whole blood by Ellman’s reagent, according to the method of Sedlak and Lindsay [25]. Both indicate the antioxidant status of the body. To measure the TNF-alpha levels and to perform histopathological analysis, samples were taken from the central part of the flap. Tissue TNF-α (one of the indicators of the inflammatory response) levels were studied by using the Rat TNF-α ELISA Kit (Abcam, Cambridge, UK) following the manufacturer’s protocol. A histopathological study of the samples was carried out by the same pathologist. The tissue samples were fixed in 10% neutral buffered formaldehyde solution and embedded in paraffin. Three-micron-thick (Microtome: Thermo Scientific Microm Hm 325) histological sections were cut, mounted on glass slides, stained with haematoxylin-eosin (HE) and evaluated by light microscope to quantify foreign body giant cells, polymorphonuclear, and mono-nuclear reactive cells. For detection of apoptosis, TUNEL was also performed.

For statistical evaluation, one-way analysis of variance (ANOVA) was used, followed by adequate post hoc tests (Dunnett’s, Sidak) for multiple comparisons. All data are represented as the mean ± SEM. The difference was considered statistically significant when p value was less than 0.05.

3. Results

The statistical analysis of the MDA levels showed significantly reduced values in the pre-ischemic trimetazidine treated group compared to the I/R group (59.84 ± 2.8 vs. 75.3 ± 6.4; p = 0.0145), which refers to smaller lipid peroxidation (Fig. 2).

Significantly higher GSH levels, both in pre- and postischemic trimetazidine treated groups (preisch. TMZ: 965.5 ± 6.3, p = 0.0035; postisch. TMZ: 1002 ± 38.6, p = 0.0002 vs. 820.9 ± 13.5) also supported an antioxidant effect of the drug (Fig. 3).

There were no significant differences in the SH- levels among the groups (control: 94.03 ± 8.584; I/R: 74.3 ± 3.763; preisch.TMZ: 98.62 ± 11.4; postisch.TMZ: 91.65 ± 6.5) (Fig. 4).

![Fig. 2. Malondialdehyde concentrations in the experimental groups. MDA serves as a marker of the lipid peroxidation. *: p < 0.05 vs. control; #: p < 0.05 between the signed groups; error bars: SEM.](image-url)
The considerable decrease of TNF-α levels in the treated groups compared to the I/R group (preisch. TMZ: 41243 ± 2183 *p* = 0.0001; postisch. TMZ: 54025 ± 5924 *p* = 0.0437 vs. 73331 ± 5762) can prove the anti-inflammatory effect of the drug (Fig. 5).

Our histopathological findings correlate with the biochemical results. Four zones are identified in all tissue samples (Fig. 6, Control). In the control group, the basic tissue structures mainly kept, oedema, necrosis or significant inflammation cannot be detected.

In the I/R group (Fig. 6, I/R) many changes can be noticed: oedema was occurring in the fatty zone and in the submuscular zone. A large number of polymorphonuclear (PMN) cells could be seen under the muscle. The muscle fibres were swollen and irregular-shaped.
Fig. 5. TNF-alpha concentrations show the grade of the inflammatory response in the investigated groups. *: $p < 0.05$ vs. control; #: $p < 0.05$ among the signed groups; error bars: SEM.

Fig. 6. Staining: HE, magnification: 5x. In the control group, the four zones can be clearly identified: A: epidermal-dermal zone; B: fatty zone; C: muscular zone, D: submuscular zone. In the I/R group oedema can be seen in the submuscular and fatty zone and the muscle fibres are swollen and irregular-shaped in the zone C. The protective function of the TMZ is well demonstrated in both (Preisch. TMZ+I/R and I/R+Postisch. TMZ) groups, showing less changes in the tissue samples: muscle fibres are approximately normal shaped, oedema and PMN-cells are barely detected in the different zones.
Fig. 7. Staining: TUNEL, magnification 10x: TUNEL staining demonstrates the apoptotic nuclei. 1. In the control group the high number of positive cells, showing up only in the follicle, are physiological - since these are holocrine glands. 2. The homogeneous positivity in the I/R group is the evidence to demonstrate the damage in the tissue, caused by the ischemia/reperfusion. 3. The protective function of the TMZ is well demonstrated in both (Preisch. TMZ+I/R and I/R+Postisch. TMZ) groups, showing barely positivity in all investigated zones.

In both TMZ treated groups tissue changes were seen to a lesser degree than in the I/R group. The muscle fibres were approximately normal shaped, oedema and PMN-cells were barely detected in the different zones (Fig. 6; Preisch. TMZ+I/R, I/R+Postisch. TMZ).

The good influence of the drug is also supported by TUNEL staining (Fig. 7). In the control group the high number of positive cells, showing up only in the follicle, are physiological - since these are holocrine glands (Fig. 7; Control). In the I/R group (Fig. 7, I/R) many apoptotic cells were found in every zone of the flap. TUNEL-positive nuclei were stained brown. This confirms that I/R also promotes the apoptosis. The TMZ management of skin flaps clearly decreased the quantity of the apoptotic cells. Apart from the epidermal-dermal zone, where apoptotic cells can be found physiologically, the number of the positive cells were considerably less in the treated groups, compared to the I/R group (Fig. 7; Preisch. TMZ+I/R, I/R+Postisch. TMZ).

4. Discussion

The use of microvascular flap transfer is very popular for defects of the whole body. It is known, that the success rate of the microsurgical vascular anastomosis, even with experienced surgeons is 90 to 95 percent, however, some severe problem such as I/R injury or the inadequate blood perfusion may still impede the complete success. I/R injury can cause severe problems in the microcirculation and it may lead to patient’s morbidity and prolonged hospitalization. The intracellular biochemical changes that
occur during the ischemic period can cause cellular dysfunction, cellular and interstitial oedema and finally can lead to cell death. Severity of these changes depend on the length of the ischemic time, since it is well known that brief ischemic condition can be protective against the negative alterations [26]. During reperfusion, following the ischemic period, reactive oxygen species are produced, which include oxygen ions, free radicals, and peroxides, all of which worsen ischemia-reperfusion damage [27, 28], impact on red blood cells micro-rheological parameters and may result in considerable disturbance of blood flow [29–31]. In the pathogenesis of I/R injury inflammation is also considered to be a critical element [32, 33].

In our study, we chose the superficial epigastric skin flap model, because it was suitable to simulate a clinical situation, that occurs when microsurgical tissue transfer is made. As Yoshida and Campos suggested the model could also simulate a vascular pedicle thrombosis, where the procedure from the diagnosis to the restoration of vascular supply could reach or exceed 6 hours, or it also can simulate a traumatic situation when replantation of amputated fingers is made [34]. In these type of models, flaps contain the epidermal-dermal zone, fatty zone, muscular zone (panniculus carnosus) and submuscular zone with a vascular pedicle of the superficial inferior epigastric artery and vein. There are controversies related to the position of the microvascular clamp. They could be used on both the artery and on the vein, or separately on the vein or on the artery to simulate different situations, which can occur in the clinical practice. Our experimental model based on superficial inferior epigastric artery and veins to reach a higher level of I/R injury and the extension of the flaps were 6.0 × 3.0 cm bilaterally.

The length of the ischemic time was based on the literature [35]; ÇetIn et al. [33]. subjected the rats to 6 hours and 10 hours of ischemia, because these time points have been reported to produce consistent biochemical, histopathological and macroscopic findings [36].

TMZ is a potent anti-ischemic drug, which decreases fatty acid oxidation and stimulates glucose utilization via the inhibition of the mitochondrial long chain 3 ketoacyl-CoA thiolase, leading to the production of adenosine triphosphate (ATP) with less oxygen consumption. It limits intracellular acidosis, decreases sodium and calcium accumulation into cells, inhibits the extracellular leakage of potassium during cellular ischemia and reduces cytolysis and membrane injury caused by oxygen free radicals. In addition, TMZ conserves mitochondrial function and energy metabolism and it is capable of inhibiting platelet adhesion-aggregation and neutrophil infiltration [19, 37, 38]. Because it does not have a negative alteration on the hemodynamic status, besides the cardiology, it also can be useful in other areas of the clinical practice.

Previously, the effect of the TMZ on the survival of skin flaps was already studied and the agent was proved to be effective. Nieto et al. investigated various pharmacological agents on the survival of skin flaps in rats. All treated groups showed a significantly greater survival of the flap than the control group. One of the best outcomes was shown in those groups receiving trimetazidine and hydralazine [39]. Kara et al. studied the effect of trimetazidine on the survival of rat island skin flaps. They compared the pre-ischemic and post-ischemic effect of the drug, and both ways seemed to be effective to improve flap survival [40].

However, this is the first study where, before the visible tissue changes, the histological and biochemical alterations were investigated after pre-and postischemic TMZ treatment in skin flaps. Blood MDA, GSH, and SH- levels and tissue TNF-α levels were evaluated for biochemical analysis. MDA is a stable product of polyunsaturated lipid peroxidation in cells, that is generated after free radical damage. GSH is one of the major endogenous antioxidants produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds. The serum levels of protein -SH in the body, can indicate antioxidant status. TNF-α is a polypeptide compound and it is an important member of the cytokine family, which plays a significant role in the regulation of the systemic inflammatory response.
In the literature, there are controversies in the administration routes and doses of this antioxidant agent [41–43]. In our study 10 mg/kg dose was chosen and the drug was administered intraperitoneally, based on some previous studies where this dose was proved to be effective [34, 44]. The timing was also different in many studies. For example, Khan and colleagues [42] published that TMZ was cardioprotective (via the activation of p38 mitogen-activated protein kinase and Akt signalling pathway) when administered at the beginning of the reperfusion period. Elimadi et al. [44] investigated the effect of TMZ on hepatic warm I/R injury, administered as an intramuscular injection with different doses (5 mg, 10 mg, 20 mg). They demonstrated that 10 mg/kg/day for 7 days before the induction of ischemia was the optimal dosage, that gave the maximal protective effects at both cellular and mitochondrial level. All these observed differences among the studies could be a consequence of different animal models, examined organs and I/R protocols. Further investigations are required to determine the optimal time and dose of administration of TMZ and to have more insight into clinical application.

In our study, we hypothesised that a single shot of TMZ will be preventive against I/R injury in epigastric skin flaps. Since in the previous studies the timing of the administration of TMZ was different, we investigated both pre- and postischemic TMZ treatment. Our data confirm the earlier findings, that TMZ has anti-inflammatory and anti-ischemic effects, independently of the timing. It could be a useful drug in the surgical practice to increase the survival time of the tissue, not just given before a planned ischemic period but also after an unexpected trauma where a reconstructive surgery is required.

5. Conclusion

TMZ is a clinically applicable and non-toxic agent, which may increase the ischemic tolerance of the tissues and can protect them from ischemia-reperfusion injury, even after an unexpected ischemic insult.

References


Pentoxifylline attenuates the local and systemic inflammatory response after infrarenal abdominal aortic ischemia-reperfusion

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Abstract.

AIMS: We studied the new anti-inflammatory effects of non-specific phosphodiesterase (PDE) inhibitor pentoxifylline (PTX) on ischaemia-reperfusion injury and postconditioning of the lower extremities. We aimed to examine the oxidative stress parameters (OSP), the inflammatory response and the changes in structure of skeletal muscle after revascularization surgery.

METHODS: 50 Wistar rats in five groups underwent a 60 min infrarenal aortic cross clamping. After the ischaemia in IR+PC group ischemic postconditioning was performed, intermittent 15 seconds reperfusion, 15 seconds ischaemic periods were applied four times. The ischemic phase was followed by a 120 min of reperfusion. In IR+PTX group the animals were treated with PTX. In IR+PC+PTX group both ischemic postconditioning and PTX treatment were performed. Blood samples and biopsy from quadriceps muscle were collected. Plasma malondialdehyde, reduced glutathione, -SH-groups, TNF-alpha, IL-6 concentrations and superoxide dismutase enzyme activity were measured.

RESULTS: The levels of OSP and the inflammatory proteins were significantly higher in the IR group. PTX treatment and PC could significantly decrease the levels of OSP and inflammatory proteins. When the animals were co-treated with PTX and PC the results were even better.

CONCLUSIONS: Inhibition of PDE by PTX could markedly decrease the inflammatory response and moderate the ischaemia-reperfusion damages after lower limb ischemia and reperfusion. Administration of PTX could potentiate the beneficial effects of PC.

Keywords: Postconditioning, reperfusion injury, vascular surgery, reactive oxygen species, TNF-alpha, leukocyte, inflammatory response, pentoxifylline (PTX)

1. Introduction

Despite significant research efforts and aggressive treatment strategies, in case of acute ischemia the extent of ischemia reperfusion injuries after revascularization surgery remains high. The severity of these injuries depends on the ischemic time, the collateral circulation of the affected limb, the localization of the occlusion and the general state of affected tissues. In reperfusion injury the developing local
than systemic inflammatory response plays a crucial role in severe tissue injury and organ dysfunction and may develop into multiple organ dysfunction syndrome-MODS. In the early reperfusion, when the molecular oxygen appears in the cell, the – xanthine oxidase catalyzed – hypoxanthine-xanthine conversion will produce a mass of superoxide radicals. Rapid generation of ROS by activated endothelial cells, neutrophils (NADPH oxidase, myeloperoxidase-MPO), lipid mediators (platelet activating factor-PAF, leukotriene B4-LTB4) are main pathways in the process of inflammatory response. During reperfusion the superoxide radicals neutralize the nitrogen monoxide-NO produced by endothelial cells. Reduced NO availability leads to augmented expression of cellular adhesion molecules, vasoconstriction, formation of micro-thrombi, induction of local inflammation, leukocyte infiltration. The nuclear factor kappa B (NFkB) is a transcription factor which determines an up-regulation of the genes responsible of the production of molecules of cellular adhesion [22]. These molecules favour the adhesion of leukocytes to the endothelium and possibly the migration within the cells [4]. These mechanisms can lead to the so-called “no-reflow phenomenon” [21].

Pentoxifyllin (1-[5-oxohexyl]-3,7-dimethylxanthine, PTX) a xanthine-derived non-specific phosphodiesterase (PDE) inhibitor, has been used for the treatment of intermittent claudication in patients suffering from peripheral and cerebrovascular disease [20]. Through its hemorheological properties, PTX can modify the conformation of red blood cells and improve the microcirculatory blood flow in chronic arterial insufficiency. PTX decreases platelet and cell aggregation and lowers the plasma viscosity. On the other hand PTX has been used in the attenuation of the inflammatory response too. Recent studies have focused on the anti-inflammatory effects of PTX, more specifically, the neutrophils. This drug improves leukocyte deformability and chemotaxis, depresses neutrophil degranulation, decreases endothelial leukocyte adhesion and lowers the sensitivity of leukocytes to cytokines. It has been reported that PTX can inhibit the production of inflammatory cytokines, and thus, reduces adhesion of neutrophils to endothelial cells and lowers the production of free radicals.

We hypothesized that single-shot, increased dose of PTX treatment in conjunction with its known hemorheological effects decreases the developing ischemia-reperfusion injury and can attenuate the local and systemic inflammatory response.

2. Materials and methods

2.1. Animal model

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

2.2. Aortic ischemia reperfusion model

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

2.3. Administration of pentoxifyllin

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

2.4. Protocol of ischemic postconditioning

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

2.5. Experimental groups

Rats were divided into five groups (10 rats in each group). In the control group a midline laparotomy was performed for three hours. Normal saline solution was administered to the animals intravenously 30 minutes before the reperfusion phase (control). The infrarenal abdominal aorta in the second group was closed for 60 minutes and then 120 minutes of reperfusion followed (IR). Rats in the third group underwent a 60 minutes of ischemia, after the ischemic phase postconditioning was performed followed by a 120 minutes of reperfusion phase (IR+PC). In the fourth group 60 minutes of ischemia was performed, 30 minutes before the reperfusion PTX was administered to the animals and then 120 minutes of reperfusion is followed (IR+PTX). Rats in the fifth group underwent a 60 minutes of ischemia, 30 minutes before the reperfusion PTX was administered to the animals, after the ischemic phase postconditioning was performed followed by 120 minutes of reperfusion (IR+PC+PTX) (Fig. 1). During the experiments there was 1 exitus in control group and 1 in IR group. In other groups there was no exitus. Peripheral blood samples and biopsy from quadriceps muscle were collected from the animals at the end of the reperfusion phase. The serum and tissue samples were harvested and stored at minus 78°C until biochemical assays.

2.6. Analysis of oxidative stress parameters

Measurement of MDA: Malondialdehyde is a marker for the quantification of lipid peroxidation in cell membranes. MDA was determined in anticoagulated whole blood, by photometric method of Placer, Cushman and Johnson [18].

Measurement of reduced glutathione and plasma thiol-groups: Reduced glutathione is the predominant low-molecular-weight thiol in cells. Because of the cysteine residue GSH is readily oxidized nonenzymatically to glutathione disulfide by electrophilic substances. GSH concentrations reduce markedly in response to protein malnutrition and oxidative stress [13]. GSH and plasma SH levels were determined in anticoagulated whole blood EDTA by Ellman’s reagent according to the method of Sedlak and Lindsay [23].
For measuring of SOD activity in serum we used Superoxide Dismutase Assay Kit (Trevigen Inc., Gaithersburg, USA), following the manufacturers protocol. This method determines the free i.e. biological active SOD activity.

2.7. Serum TNF-alpha and IL-6 quantification

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

2.8. Histological examinations

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

The biopsies were made with the following method:

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

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

3. Results

3.1. Plasma malondialdehyde levels

We measured in an in vivo animal model the values of malondialdehyde plasma-level indicating membrane damage and lipid peroxidation. MDA concentration was significantly higher in all groups (IR, IR+PC, IR+PTX, IR+PC+PTX) comparing to the control group (79.39 ± 0.64; 68.16 ± 0.62; 70.97 ± 1.23; 65.52 ± 0.98 nmol/ml vs. 61.12 ± 1.75 nmol/ml/$p < 0.0001$; $p = 0.002$; $p < 0.0001$; $p = 0.0285$). Our data showed significantly lower MDA concentrations in IR+PC, IR+PTX and IR+PC+PTX groups comparing to the IR group (68.16 ± 0.62; 70.97 ± 1.23; 65.52 ± 0.98 nmol/ml vs. 79.39 ± 0.64 nmol/ml/$p < 0.0001$; $p < 0.0001$; $p < 0.0001$). In the IR+PC+PTX group we found significantly lower MDA concentrations than in IR+PTX group (65.52 ± 0.98 nmol/ml vs. 70.97 ± 1.23 nmol/ml/$p = 0.0065$) (Fig. 2).

3.2. Reduced glutathione levels (GSH)

The values of reduced glutathione levels were significantly lower in two groups (IR, IR+PTX) comparing to the control group (725.1 ± 11.26; 808.6 ± 14.72 nmol/ml vs. 877.1 ± 20.7 nmol/ml/$p < 0.0001$; $p = 0.033$). Our data showed significantly higher concentrations in IR+PC,
IR+PTX and IR+PC+PTX groups comparing to IR group (822.8 ± 23.13; 808.6 ± 14.72; 830.6 ± 17.3 nmol/ml vs. 725.1 ± 11.26 nmol/ml/p = 0.0018; p = 0.0097; p = 0.0007) (Fig. 3).

3.3. Plasma thiol groups (–SH)

We detected in the IR group significantly lower level of –SH comparing to control group (42.09 ± 2.15 nmol/ml vs. 54.02 ± 2.68 nmol/ml/p = 0.003). There was no significant difference in –SH level between other groups (Fig. 4).

3.4. Enzyme activity of superoxide dismutase (SOD)

We have detected in two investigated groups significantly elevated (IR+PC, IR+PC+PTX) and in one group significantly lower (IR) SOD activity comparing to the control group (1088 ± 42.1; 1113 ± 52.8 U/l vs. 893.7 ± 32.6 U/l/p = 0.0026; p = 0.0006; 533.8 ± 17.4 U/l vs. 893.7 ± 32.6 U/l/p < 0.0001).

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

Fig. 4. Concentrations of –SH groups in the plasma. *:p < 0.05 vs. control; error bars: SD.
In IR+PC, IR+PTX and IR+PC+PTX groups we have detected significantly elevated SOD activity comparing to IR group (1088 ± 42.1; 952.4 ± 34.1; 1113 ± 52.8 U/l vs. 533.8 ± 17.4 U/l/p < 0.0001 in all three comparisons). In IR+PC+PTX group we found significantly elevated SOD activity than in IR+PTX group (1113 ± 52.8 U/l vs. 952.4 ± 34.1 U/l/p = 0.02) (Fig. 5).

3.5. Serum TNF-α levels

In the study we measured the TNF-α levels in the groups. The values were significantly higher in IR group than in the control group (21.9 ± 0.49 pg/ml vs. 18.4 ± 0.3 pg/ml/p < 0.0001). In IR+PC, IR+PTX and IR+PC+PTX groups we detected significantly lower values comparing to the IR group (19.7 ± 0.3; 18.6 ± 0.4; 19.05 ± 0.3 pg/ml vs. 21.9 ± 0.5 pg/ml/p = 0.0002; p < 0.0001; p < 0.0001) (Fig. 6).

3.6. Serum interleukin-6 (IL-6)

We investigated the serum IL-6 levels in our groups. The values were significantly higher in IR group, than in the control group (144.3 ± 4.2 pg/ml vs. 109.3 ± 1.9 pg/ml/p = 0.002). We have found

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

**Fig. 6.** TNF-alpha concentrations shows the grade of inflammatory response in the groups. *:*p < 0.05 vs. control; #:#p < 0.05 between the signed groups; error bars: SD.
significantly lower concentrations in IR+PTX, IR+PC and IR+PC+PTX groups than in IR group (112.9 ± 2.1; 119.9 ± 3; 115.9 ± 2.7 pg/ml vs. 144.3 ± 4.2 pg/ml/p < 0.0001; p < 0.0001; p < 0.0001) (Fig. 7).

3.7. Histological results (Fig. 8)

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

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

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

Fig. 8. Quadriceps muscle slices, HE, 200x. Control group: Healthy muscle tissue. IR group: Muscle fibres are swelled. Focal atrophy and necrosis can be seen. IR+PC group: Healthy muscle structure, the fibers are gently swelled and the interstitium is splayed. IR+PTX group: Healthy muscle structure, the fibers are gently swelled. IR+PC+PTX group: The muscle structure is kept, healthy.
In the IR+PC group the basic muscle structure is mainly kept. Muscle fibres are gently swelled but interstitial edema or necrosis cannot be defined (IR+PC).

In the slice of the IR+PTX group the muscle structure is undamaged, healthy, there is no necrosis or atrophy in the fibres (IR+PTX).

In the IR+PC+PTX group potentially healthy muscle structure can be seen. Edema or necrosis cannot be defined (IR+PC+PTX).

4. Discussion

After revascularization procedures we always have to face with severe or less reperfusion injury. Numerous factors can modulate the extent of reperfusion injury including inflammatory response. Among the outcomes of reperfusion injury are included: (I.) endothelial and vascular dysfunction and the sequels of impaired arterial flow, which may concur with the ‘no-reflow phenomenon’; (II.) metabolic and contractile dysfunction; (III.) arrhythmias in case of myocardial I/R; (IV.) cellular death by cellular swelling, and apoptosis. These processes lead to changes of hemorheological environment and these changes may be harmful for red blood cells, impairing their deformability and influencing their aggregation behavior [16]. Ischemic pre-, postconditioning and remote conditioning are well known methods for reducing ischemia-reperfusion injury. These methods can initiate pathways which lead to attenuation of superoxide anion generation by activation of neutrophils and endothelial cells, and activation of mitochondrial KATP channels via adenosinergic G protein-coupled receptor activation. Better endothelial function increases NO release by endothelial cells, which further attenuates superoxide anion levels and both neutrophil activation and adherence to the endothelial cells. Postconditioning decreases the intracellular buildup of oxidants and calcium in cardiomyocytes, which inhibits mPTP opening, thereby inhibiting both apoptosis and necrosis [25]. Recently Grau et al. reported that remote preconditioning increases red blood cell deformability through red blood cell-nitric oxide synthase activation [9]. Nemeth et al. investigated simultaneously the hemodynamic, microcirculatory and arterio-venous micro-rheological parameters in infrarenal or suprarenal aortic cross-clamping model in the rat [17]. Recently reported examinations investigated the beneficial effects of improvement of microcirculatory system (contractile recovery) after reperfused acute myocardial infarction [2] and in experimental intestinal ischemia/reperfusion [28]. So these processes cannot be separable from each other. Change in microcirculatory system and hemorheological environment are parts of defense against reperfusion injury.

PTX a xanthine-derived non-specific PDE inhibitor, has been used for the treatment of intermittent claudication in patients suffering from peripheral and cerebrovascular disease [19]. Through its hemorheological properties, PTX can modify the conformation of red blood cells and improve the microcirculatory blood flow in chronic arterial insufficiency. PTX decreases platelet and cell aggregation and lowers the plasma viscosity. PTX can improve the microcirculatory parameters in cerulean-induced acute pancreatitis in rat [29].

On the other hand, recently PTX has been used in the attenuation of the inflammatory response too. PTX can decrease the inflammatory process after cardiopulmonary bypass in open-heart surgery, sepsis, and acute respiratory distress syndrome (ARDS) in neonates. PTX exerts multiple beneficial effects on the inflammatory cascade by increasing intracellular cyclic adenosine monophosphate (cAMP) and decreasing TNF-alpha and IL-6 synthesis [5, 24]. An increase in cAMP levels in muscle fibers results in the activation of protein kinase-A-PKA and facilitates synaptic transmission in the mammalian neuromuscular junction (NMJ). Blocking the production of TNF-alpha by PTX takes place by activation of adenylyl cyclase and increased levels of intracellular cAMP. This in turn decreases the amount of arachidonic acid that undergoes peroxidation. The overall effect
is a decrease in systemic and local concentrations of inflammatory agents such as cyclooxygenase [1, 15].

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

The relationship between transcription factors and PTX has yet to be determined. PTX dose-dependently reduced NFkB subunit nuclear translocation when given lipopolysaccharide (LPS) [10]. PTX also diminishes NFkB translocation in activated T lymphocytes [27]. These results suggest that PTX is involved in a common signaling pathway, however, further experimentation is necessary.

In our study we hypothesized that single shot, increased dose of PTX treatment in conjunction with its known hemorheological effects decreases the extent of developing ischemia-reperfusion injury and can attenuate the local and systemic inflammatory response. A recent study has demonstrated that PTX attenuates ischemia reperfusion injury in skeletal muscle and other tissues by decreasing neutrophil adhesion to endothelial cells, ROS production, and platelet activation (PAF) [12]. During investigation of oxidative parameters we have found that postconditioning and PTX administration decreased significantly the plasma levels of MDA comparing to the IR group which further decreased in the “co-treated” group. GSH is an endogenous antioxidant. Postconditioning and administration of PTX could significantly moderate the decrease of GSH level in the groups. Enzyme activity of SOD was significantly higher both postconditioned and PTX-administered groups comparing to IR group. Beside the hemorheological effects, the additive beneficial pathway of PTX can be the anti-inflammatory effect. Recently El-Ghoneimi et al. [6] reported significantly lower levels of serum TNF-alpha and a lower necrotic area in liver tissue in the PTX group. PTX has been shown to downregulate the synthesis of proinflammatory mediators like IL-6, improve microvascular hepatic and intestinal blood flow after hemorrhagic shock [7, 11, 14, 26]. During investigation of inflammatory response we performed TNF-alpha and IL-6 ELISA. We found that administration of PTX could decrease significantly both the TNF-alpha and the IL-6 concentrations in plasma. The degree of these decreases could beyond the decrease observed in the postconditioned groups. Our data seems to be confirmed the recent findings, that PTX has anti-inflammatory effects through inhibition of TNF-alpha and IL-6 formation and attenuation of neutrophil adhesion to endothelial cells and platelet activation. As TNF-alpha is an inducer of the inflammatory cascade, it also acts as trigger to the extrinsic pathway of apoptosis [3, 8]. So decreased TNF-alpha concentration can lead to attenuation of apoptosis as well.

5. Conclusion

Our results showed that administration of PTX can decrease the extent of ischemia reperfusion injuries including the inflammatory response through its hemorheological- and recently described anti-inflammatory effects. In our study the administration of PTX could reach almost the same protection like ischemic postconditioning. The results of the investigated inflammatory mediators could support the finding, that PTX has anti-inflammatory or immunmodulating effects as well. So the clinical importance of this investigation is the possible beneficial effects of PTX on ischemia-reperfusion injury due to its hemorheological and anti-inflammatory effects.

Acknowledgments

This work was supported by the Hungarian Science Research Fund OTKA-K108596.
References


