Investigations of pathological conditions and circulation during oncological reconstructive surgeries

PhD thesis

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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. The four most common cancers occurring worldwide are: lung, female breast, colorectal and prostate cancer. These four account for around 4 in 10 of all cancers diagnosed worldwide.

In our study we mainly focused on breast and head/neck (oesophagus, hypopharyngs) cancers. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, 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).

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 the cancer or for the detection of a second tumour. There are a lot of attempt to find new markers, but still, it is very important to do researches on this field and improve the diagnostic tool for cancers.

Beside the early diagnosis and adequate therapy, reconstruction of the defects after oncological ablative surgeries is also a big challenge for the plastic-reconstructive surgeons. 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. Although, the success rates of free tissue transfer 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. For these reasons the detection of biochemical changes and microcirculatory disorders in flaps during ischemia-reperfusion (I/R), are of high importance.

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. 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 complement system and mitochondria can influence the severity of the IRI**.** During ischemia, the metabolism shifts towards the anaerobic, which results in a decrease in cell pH. To buffer this accumulation of H^+ ions, the Na^+/H^+ exchanger excretes excess hydrogen ions, which leads to a large influx of sodium ions. Ischemia also depletes cellular

adenosine-tri-phosphate (ATP), which inactivates ATPases (e.g., Na^+/K^+ ATPase, Ca^{2+} ATPase), reduces active Ca^{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. Although prompt reperfusion restores the delivery of oxygen and substrates required for aerobic ATP generation and normalizes extracellular pH by washing out accumulated H^+, 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.

1.1.1 Trimetazidine

Trimetazidine (TMZ), is a well known anti-ischemic drug, which so far clinically is 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
- limits intracellular acidosis, reduces sodium and calcium accumulation into cells
- inhibits the production of deleterious lipid metabolites
- inhibits mitochondrial permeability transition-pore opening and protects tissues from prolonged ischemia-reperfusion injury.
- decreases cytoliysis and membrane injure caused by oxygen free radicals
- attenuates the inflammatory response and reduces the rate of apoptosis expression

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. 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.

2 AIMS

We planned to perform three major investigations. In the first study we focus on a new diagnostic opportunity of breast cancers. In the second and third study, the possibilities of the reduction of ischemia-reperfusion injury during reconstructive free flaps 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. First aim of the **second study** is to demonstrate, that measurable injury caused by ischemia-reperfusion, occuring 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 hemorrheological 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 (preischemical and postischemical), 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 any 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.

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

The expression pattern of IF proteins is tissue specific and developmentally regulated. The expression of specific subsets of IF proteins classically serves 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.

Antal Tapodi, Daniel M. Clemens and co-workers examined the original role of the BFSP1 in 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.

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 of participating in the experiment was 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 were 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. 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 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 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.

3.3 RESULTS

3.3.1 Western blotting

We examined human ex vivo clinical samples, precisely breast cancer tumour samples from cancer patients. In this study we have proved with immuno blotting, that BFSP1 is present in tumour samples. 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 type of the antibodies.

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 provide a good background for our further investigations with serum.

3.3.2 Immunohistochemistry

For the identification of BFSP1 protein in the tumour samples immunohistochemistry was performed with S38 anti- BFSP1 antibody. First, we examined tumour-free (behind the nipple) tissue area from different treated patients. In this case only the ducts, ductules and acini were stained positively with the antibody, but there

were no positive cells in the surrounding tissues. The amount of positive staining cells was not influenced by the different treatments. Regarding with those area where the tumour could be detected the results were different according the treatments. In those cases, where the patient received neoadjuvant therapy, and the tumour was regressed, the amount of the positive cells was significantly decreased compared to those cases, where the tumour was still presented (even if the patient got neoadjuvant therapy).

3.4 DISCUSSION

In the case of breast cancer (BC) 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. Absence of these receptors is a predictor of a poor prognosis. Furthermore, today, they are also used and suitable to select hormone therapy. 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. 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.

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 provide and maintain cell shape and structure, and are key to important cellular events, including cell division, movement and vesicular transport. 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 fiber specific proteins (BFSPs) belong to the family of intermediate filament proteins (IF). BFSP1 or filensin is expressed in lens fiber cells after differentiation has begun. This protein functions as a component of the beaded filament which is a cytoskeletal structure, found in lens fiber cells. 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. 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. The critical role of these proteins is mainly emphasized by the presence of cataracts.

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, a 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 we could confirm that there is a significant difference in the contents of BFSP1 according to the presence of tumour. In those patients, who did/did not receive neoadjuvant therapy but/and the tumour was presented macroscopically, the amount of the positive staining cells increased considerably, comparing with the tissue samples of those patients who received neoadjuvant therapy and the tumour was regressed. Based on our results it seems that the BFSP1 protein is sensitive enough to indicate the tumour cells in the case of ductal breast carcinomas.

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 provide 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 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 the 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. 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.

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 experimental studies to protect different organs (myocardium, intestine, liver, and kidney) from the ischemia-reperfusion injury. Numerous evidences exist, 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 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. 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.

4.2.3 Surgical procedure

The rats were 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 and scrubbed with betadine. Then 3x6 cm flap was created on both sides of the abdomen. In our study, the epigastric flap was chosen. 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 vascular patency tests w 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 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 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 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 (Terminal deoxynucleotidyl transferase dUTP nick end labelling) 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, which is an important determinant of the microcirculatory pattern, with LORCA ektacytometer. 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, Lineweaver-Burke nonlinear curve fitting technique was used to calculate the maximal EI (EImax) value at extrapolated infinite shear and the shear stress value required for half of EImax (SS1/2). Measurements were performed at 37°C.
Blood samplings were performed from lateral tail veins, and hemorheological examination were 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 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 Malondyaldehid

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 significant decrease was shown only in that

group where the TMZ was administered prior to the ischemia. The results of the treated groups were nearly as good as in the control group.

4.3.1.2 Reduced Gluthation

GSH levels were reduced in all groups comparing to the control. Significantly higher GSH levels were measured both in pre-and postischemic trimetazidine treated groups compared to the I/R group, which supported the antioxidant effect of the drug.

4.3.1.3 Sulfhydril group (SH-)

There were no significant differences in the SH- levels among the groups.

Changes of TNF-α level in skin sample

Comparing to the control group, except the Preisch. TMZ+ I/R group, significantly higher values were measured. Considerable decrease of TNF- α levels in the treated groups were noticed, compared to the I/R group.

4.3.2 Histopathological results

4.3.2.1 Hematoxylin-eosin

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

In the I/R group many changes can be noticed: oedema was occurring in the fatty zone and in the submuscular zone. 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 less 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.

4.3.2.2 TUNEL- staining

The good influence of the drug is also supported by TUNEL staining. 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. In the I/R group 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 less in the treated groups, compared to the I/R group.

4.3.3 Changes in hemorheological parameters

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.

4.4 DISCUSSION:

The use of microvascular flap transfer is very popular to reconstruct the defects of the whole body. 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 oedema and finally can lead to cell death. 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. 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, impact on red blood cells micro-rheological parameters and may result in considerable disturbance of blood flow. In the pathogenesis of I/R injury inflammation is also considered to be a critical element.

In our study, we chose the superficial epigastric skin flap model. 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; ÇetIn et al. 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.

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 $1^{st} - 4^{th}$ 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 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 signalling 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 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.

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 unambigous difference between the preischemical and postischemical TMZ administration. The benefit 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

Jejunal flaps are commonly used for reconstruction of the esophagus after cancer resections. Their main advantages are the followings: tubular structure similar to the esophagus, available length, production of mucus, lack of functional gastrointetinal complication after removal, peristaltic activity similar to the pharyngoesophagus and the ease of preoperative preparation. Besides the esophagus, jejunal flap is also suitable for vaginal reconstruction for example, in the case of: congenital absence of vagina or after surgical resection of tumors. In these cases jejunum transfer is a primary choice contrary to other intestinal transfers, particularly because it causes fewer defecation problems. 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. 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. In spite of several suggested strategies/methods, so far effective, widely used method is not clinically available as a solution of this problem.

Ischemic preconditioning (IPreC) has been proved to produce resistance to the loss of blood suply and this method is able to improve the survival of tissue, subject to global ischemia. During IPreC brief period of ischemia followed by reperfusion is used, which increase the ischemic tolerance of the tissue 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. Main advantage of this method, that it is also able to reduce the degree of the damage even after an unexpected event.

Our aim was to investigate the results of chemical preconditioning and postconditioning with trimetazidine, in jejunal-flap model in 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 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 forth group (IPreC+ I/R; Group 4) ischemic preconditioning was used: 2x5 (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.

In the second part of the study (B) we compared the effect of IPostC with the TMZ postconditionig, and the additive effect of these two methods were 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 ishcemic postconditioning was also performed, as mention above.

Animals, in the TMZ 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.

5.2.2 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 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. Then according 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 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.

5.2.3 Biochemical analysis

5.2.3.1 Oxidative stress parameters

MDA, GSH, SOD and SH- levels were measured from the serum. 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.

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

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).

5.2.4 Histopathological analysis

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 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.

According 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 staining was also performed.

5.2.5 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 *p* value was less than 0.05.

5.3 RESULTS

5.3.1 Biochemical results of the comparison of TMZ and IPreC treatment

Reduced Gluthation (GSH) and Malondialdehyde (MDA)

The GSH levels were significantly lower in all groups compared, to the control group. Compared to the I/R group, significantly higher values were measured in all treated groups ($p<0,0001$ in all cases).

The MDA levels were considerably higher in all groups compared to the control group. 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.

Sulphhydril groups (SH-) and superoxide dismutase enzym activity (SOD)

SH-levels were also reduced in all groups comparing to the control. In those groups where TMZ was administered the SH- levels were significantly higher than in I/R group. Furthermore, in the IPreC group, the SH- levels were considerably decreased compared to that group, which received the TMZ before the ischemia.

The SOD enzym 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.

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

Inflammatory cytokines: TNF-alfa, IL-6

The TNF-alfa levels were elevated in all groups compared to the control. 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 in the last group (combined treated) did not show significant elevation compared to the control. 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 more effective method.

5.3.2 Biochemical results of the comparison of TMZ and IPostC treatment

Reduced Gluthation (GSH) and Malondialdehyde (MDA)

The GSH levels were significantly lower in all groups, except the last one, compared, to the control group. Compared to the I/R group, significantly higher values were measured in all treated groups. 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. 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

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.

Sulphhydril groups (SH-) and superoxide dismutase enzym activity (SOD)

SH-levels were also reduced in all groups comparing to the control. In those groups where TMZ was administered 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.

The SOD enzym activity was significantly lower in all groups compared to the control, and significantly higher in all treated groups comparing to the I/R group ($p<0,0001$ in all groups), although the values of IPostC group was less elevated than the TMZ treated groups. The best result was in that group, where the TMZ and IPostC were also applied.

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

Inflammatory cytokines: TNF-alfa, IL-6

The TNF-alfa levels were elevated in all groups compared to the control. 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 and IPostC group.

The results of the IL-6 were similar to the TNF-alfa level, regarding the and I/R groups. Among the treated groups, significant difference was measured only between the TMZ before rep. group and the IPostC group.

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.

5.3.3 Histopathological results

5.3.3.1 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 distinguish 6 grades from eachother, from grade 0-grade 5. Grade 0 represents the normal mucosal villi, and, in grade 5 disintegration of lamina propria, haemorrhage and ulceration can occur. In the control group, we can see a normal mucosal villi, without any signs of the mucosal injury. In our study the nontreated ischemic-reperfusion group (I/R) was between the grade 4 and 5 with denuded villi and disintegration of lamina propria. Administration of TMZ before ischemia was more effective than the administration of the drug before the reperfusion period. According 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. Based on our histopathological results, the best outcome was in the last group where both TMZ and IPostC was used. Furthermore, the additive effect of TMZ and IPostC was more stronger than TMZ and IPreC.

5.3.2.2. TUNEL-staining

In the control group positive cells (staining brown) are 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 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 the number of the positive cells in 8 crypts (from a representating 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, considerable decrease was detected in all cases, however the most significant reduction was found in the combined treated groups.

5.4 DISCUSSION

Jejunum is one of the most frequently used free flap in the head and neck region for pharynx and cervical esophagus reconstruction, but it is also a suitable flap for the reconstruction of the vagina. However, the jejunum is one of the most sensitive tissue 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. Ischemic injury has been implicated as one of the most important etiologic factor 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. 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. 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, endothel barrier dysfunction, increased recruitment of leucocytes and macrophages, nuclear transcription factor κB activation and consquent 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.

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 pior, or right after the prolonged ischemic insult, providing protection against IRI. There are a lot of study, 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.

Trimetazidine (TMZ), [1-(2,3,4 trimetoxibenzyl)-piperazine 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.

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. 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, which describes the morphological changes and mucosal damages, associated with IRI in the intestine. 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. 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 measurment 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 dosis of TMZ was 10 mg/kg, based on previous studies. We chose the 40 minutes of ischemia beacuse 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 dissapear, if the reperfusion period is long enough. Therefore, we preferred to use only 60 minutes of reperfusion in our study.

In an other study Yalcin et al. 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 were 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 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.

Based on our study, the treatment with TMZ is promising, and it could be an 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 the 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

1.) 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 not only presented in the eye lens but also in ex vivo human breast cancer.
   b. The same BFSP1 protein occurs 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 the 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.

2.) In the **second and third** study we demonstrated the effect of trimetazidine (TMZ) against ischemia-reperfusion injury in skin flaps and, in jejunum. According 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 amount of apoptotic cells.
   d. There is no unambiguous difference between the preischemical and postischemical 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 antiischemic effect of ischemic pre- and postconditioning. This effect is more stronger, when the TMZ is applied together with IPostC.

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8 LIST OF PUBLICATION

8.1 TOPIC RELATED JOURNAL ARTICLES

- **Petrovics L**, Nagy T, Hardi P, Bognar L, Pavlovics G, Tizedes Gy, Takacs I, Jancso G: The effect of trimetazidine in reducing the ischemia-reperfusion injury in rat epigastric skin flaps. CLINICAL HEMORHEOLOGY AND MICROCIRCULATION &: p. &. (2018)
  (IF (2018): 1,914)

- Nagy T, Hardi P, Takacs I, Toth M, **Petrovics L**, Jancso G, Sinay L, Fazekas G, Pinter O, Arato E: Pentoxifylline attenuates the local and systemic inflammatory response after infrarenal abdominal aortic ischemia-reperfusion. CLINICAL HEMORHEOLOGY AND MICROCIRCULATION 65:(3) pp. 229-240. (2017) **(IF (2017): 1,679)**

8.2 TOPIC RELATED SCIENTIFIC PRESENTATIONS

- **L. Petrovics**, I. Takacs, G. Jancso, T. Nagy, P. Hardi, F. Németh, Z. Trojnar, 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)**
- G. Jancso, I. Takacs, P. Hardi, B. Gasz, T. Nagy, L. Bognar, **L. Petrovics**, P. Varga, E. Arato, L. Sinay: Ischemic postconditioning decreased inflammatory response and oxidative stress in reperfusion injury evoked by aorto-bifemoral bypass surgery. **53rd Congress of the European Society for Surgical Research (**ESSR **2018) Madrid, Spain;** 05.30-06.02.2018 **(Poster)**
- **Petrovics Laura**, Nagy Tibor, Hardi Péter, Németh Franciska, Trojnár Zoltán, Takács Ildikó, Pavlovics Gábor, Jancsó Gábor: Iszkémia-reperfúziós károsodások csökkentése ismert anti-iszkémiás szerrel, patkány bőrlebenyekben,
  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, **Petrovics L**.: The role of trimetazidine in reducing ischemia-reperfusion injury in rat epigastric skin flap model, HMAA, Balatonfüred, 2017. augusztus 25-26
- Németh F., Trojnár Z, **Petrovics L.:** Trimetazidin szerepe az iszkémia-reperfúziós károsodások csökkentésében, patkány bőrlebenyek esetén, Grastyán Konferencia, Pécs, 2017.03.29-31