SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The effects of early and delayed remote ischemic preconditioning in partial liver ischemia-reperfusion in rat model

by Zsuzsanna Sarolta Magyar, MD

Supervisor: Prof. Norbert Németh, MD, PhD, DSc

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF CLINICAL MEDICINE

DEBRECEN, 2019

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Doctoral School of Clinical Medicine, University of Debrecen

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The PhD Defense takes place at the Lecture Hall of Building „A”,
Department of Internal Medicine, Faculty of Medicine, University of Debrecen,
2:00 pm, 5th of September, 2019.

1. INTRODUCTION

Partial or complete liver ischemia occurs in many pathophysiological conditions, such as trauma, shock or during operations on the liver and during liver transplantations. In many situations the clamping of the portal triad is essential to perform these operations, which cause ischemia-reperfusion injury.

Based on the literature it is well known that the ischemia-reperfusion (I/R) injury can cause serious tissue damage, which can influence the postoperative morbidity and mortality. During ischemia the metabolic balance of the tissues is overturned, causing tissue damage. As a consequence of hypoxia and acidosis, the intracellular pH is decreased and the lactate is accumulated. After the ischemic insult, the restarting of the blood flow paradoxically enhances the damage and induces further inflammatory reactions. Free-radicals and mediators released from the process are influencing the microcirculation. There is a strong relationship between the micro-rheological parameters and the microcirculation, furthermore the metabolic changes and the oxidative stress may have an effect on the red blood cell aggregation and red blood cell deformability.

After the I/R injury occurring during the surgery of parenchymal organs, the hemorheological parameters also show a change. The consequential micro- and macro-rheological differences lead to changes in microvascular state and decreased perfusion. The decreased red blood cell deformability may result increased blood viscosity. The enchanced red blood cell aggregation causes elevated blood viscosity and increases the flow resistance. During the research of pathophysiological changes occurring by surgical interventions, it is also important to know the changes in hemorheological parameters.

Regarding the fact, that ischemia-reperfusion may occur in many pathophysiological conditions and during surgical interventions, intensive researchs are in process to develop a pharmacological agent or intervention, that could be effective in the prevention and reduction of tissue damage caused by

I/R. One method of increasing the ischemic tolerance of the tissues is the remote ischemic preconditionig (RIPC). The process means intermitted short-term interruptions of the blood flow on an organ or extremity prior to another organ's manifest ischemia-reperfusion. The protective benefits of RIPC have been already proved in experimental animals, but the therapeutic efficiency in humans is still unclear.

However the patomechanism of the process is not fully known. There is no consensus the number and duration of the ischemic cycles, and the optimal time of the preconditioning before ischemia-reperfusion. The adequate evaluation of experimental results can significantly contribute to understanding the pathological changes, that cause micro-rheological and microcirculatory disturbances and to develop a suitable therapeutic strategy.

2. AIMS AND OBJECTIVES

1. Our aim was to develop an animal model where the effects of partial liver ischemia-reperfusion can be investigated.

2. In our experiment, we wanted to examine the effects of the 60-minute 70% partial liver ischemia and 120 minutes of reperfusion on vital parameters, hemodynamic status, microcirculation, acid-base-, hematological-, and micro-rheological parameters in a rat model.

3. The aim of our experiment was to analyze the morphological changes resulting from ischemia-reperfusion and to investigate the relation of histological differences with microcirculation.

4. We aimed to compare the effects of the early and delayed remote ischemic preconditioning groups to the group undergoing ischemia-reperfusion.

3. MATERIALS AND METHODS

3.1. Experimental animals, operative technique and sampling protocol

The experiment was approved and registered by the University of Debrecen Committe of Animal Welfare (Permission Nr.: 20/2011; 25/2016 UDCAW), in accordance with the Hungarian Animal Protection Act (1998. XXVIII. Law) and the EU (2010/63 EU-Directive) regulations.

Twentyone male and female Crl:WI rats (Toxicoop Ltd., bodyweight: 386.5±57 g) were randomly divided in three experimental groups, there were 5 male and 2 female rats in each group. The experimental animals were housed in cages, at room temperature, received standard rat food and water ad libitum. The age can be a crucial factor during operations, therefore the experimental rats were selected from the same age (14-16 weeks old). One day before the operation all animals received Flunixin (2.5 mg/kg s.c.) as analgesia. The preconditioning protocols and the surgical interventions were performed in general anesthesia (60 mg/kg Thiopental, i.p.). As premedication the animals received atropin-sulphate (0.06 mg/kg, s.c.). The animals were placed in a supine position on a heated pad to maintain permanent body temperature.

In the Control group (n=7) the left inguinal region and the abdomen of the experimental animals were completely shaved, and disinfected with Betadine. In left inguinal region 1 cm long horizontal incision was performed, then the femoral artery was preparated and cannulated (BD Neoflon^{TM} 26G) under operating microscope (Leica Wild 64 M650). Midline laparotomy was performed from the xiphoid process to the pecten pubis bone, the intestines were placed on the left side of the animal, covered by a plastic bag to avoid dehydration. 70% partial liver ischemia was achived by vascular occlusion of the biliovascular pedicle. Atraumatic, microvascular clip was applied for 60 minutes on the pedicle, while the caudate process and the right inferior and superior lobes were kept intact. The change in the color of liver lobes indicated

the success of the occlusion. After removing the microsurgical clip 120 minutes reperfusion period was observed. During the 60-minute ischemic and 120-minute reperfusion period, the abdominal wound was covered in two layers. As a first layer, a plastic sheet was used to minimize the fluid loss due to evaporation. As a second layer, a gauze sheet was placed on the abdomen to reduce the heat loss.

In the preconditioned groups three cycles of 10-minute ischemia interrupted by 10-minute reperfusion periods were provided. A tourniquet was applied around the left thigh above the inguinal ligament to induce ischemia. Preconditioning was applied either 1 hour (RIPC-1 group, n=7) or 24 hours (RIPC-24 group, n=7) before liver ischemia.

Before the operation (Base), and at the 30^{th}, 60^{th} és 120^{th} minute of reperfusion (R-30, R-60, R-120) blood samples were taken from the cannulated femoral artery for laboratory measurements (Anticoagulant: K_3-EDTA 1.5 mg/ml)(~0.4-0.5 ml). After each blood sampling physiological saline solution was administrated.

Respiratory rate, rectal temperature, organ surface temperature (ischemic- and non-ischemic liver lobe), microcirculatory (ischemic- and non-ischemic liver lobe, jejunum, right kidney) and hemodynamic measurements were perfomed before the operation (Base), at the 60^{th} minute of ischemia (I-60), and at the 30^{th}, 60^{th} és 120^{th} minute of reperfusion (R-30, R-60, R-120). At the end of the examined period tissue was harvested from the liver and the experimental animals were euthanised by exsanguination.

3.2. Respiratory rate, temperature

The respiratory rate was regularly determined to follow the general state of the experimental animal. To determine the rectal temperature, a digital thermometer was used and for measuring surface temperature values an infrared thermometer was apllied (Rudolf Riester GmbH, Germany).

3.3. Hemodynamic parameters

For indirect monitoring of blood pressure and heart rate, Apollo Amplifier non-invasive blood pressure monitor (IITC Inc., USA) was used, for which a cuff was placed on the tail of the experimental animal.

3.4. Microcirculatory measurements

Laser Doppler flowmetry (LD-01 Laser Doppler tissue flowmeter, Experimetria Ltd., Hungary) was applied for monitoring the changes in the microcirculation.

Laser Doppler flowmetry is a non-invasiv, widely used instrument for monitoring the changes in the microcirculation and viability of the tissue. The method is based on the Doppler frequency shift phenomenon. The wavelength of the emitted, coherent light show changes when it meets moving particles, but it stays unchanged if encounters static elements. The emitted laser beam is reflected from the surface of the moving red blood cells and show changes in the wavelength. The reflected light beam is detected by the device, and this shift is displayed on the computer screen. The great advantage of this method is that it is real-time, the result is directly appreciated, allowing immediate action.

The wave pattern of the laser Doppler flowmetry can be influenced by numerous factors, such as temperature, metering instability, respiratory and muscle movement, tissue dryness, local blood pressure, oxygenation, hemolysis, serum lipid status.

The standard pencil probe of the instrument (MNP100XP, Oxford Optronics Ltd., United Kingdom) was placed on the ischemic- and non-ischemic lobe of the liver, on the antimesenteric surface of the jejunum, and on the anterior surface of the right kidney.

The instrument determines the blood flux unit (BFU), which is relative, dimensionless number. The degree of change is directly proportional to the

number and speed of the moving red blood cells, however independent of the direction of their movement.

After stabilizing the signal the curve is registrated to determine the BFU of the 20 second long examining period, which is obtained after off-line analysis.

3.5. Laboratory measurements

3.5.1. Acid-base parameters

The acid-base parameters were measured by a portable, wireless, hand-held EPOC® Blood Analysing System device (Siemens Healthcare GmbH, Germany), and the blood gas parameters, electrolyte and metabolic values were analyzed. We need approximetaly 0.1 ml blood sample for each measurement. In our experiment blood pH, lactate concentration, pO_2 and pCO_2 values were determined.

3.5.2. Hematological parameters

The qualitative and quantitative hematological parameters (RBC [T/l], Hgb [g/dl], Hct [%], MCH [pg], MCV [fl], MCHC [g/dl], WBC [G/l], Plt [G/l]) were determined by Sysmex F-4500 (TOA Medical Electronics Corp., Ltd., Japan) microcell counter. The test requires 70 µl blood for the measurements. After the aspiration of the blood the device determines the hemoglobin concentration (Hgb [g/dl]) by fotometric method and based on the cellular separation on the impedance principle, give the parameters derived from these (leukocytes: ~ 30-300 fl, erythrocytes: ~ 25-250 fl, thrombocytes: ~ 2-30 fl).

3.5.3. Red blood cell deformability

The red blood cell deformability was measured by LoRRca MaxSis Osmoscan ektacytometer (Mechatronics BV, The Netherlands), where the

elongation of the red blood cells were continuously analyzed in a controlled shear stress range (shear stress, range: 0.5-30 Pa) by laser diffraction method.

Laser diffraction ellipsometry or ektacytometry is a widely used method for examining the deformability of red blood cells. During the red blood cell deformability measurments in the function of shear stress the elongation index profile can be detected, where the higher elongation index values reflect the well deformabled red blood cells.

The suspending medium is a very important component fot the measurements, since a high viscosity medium is needed to transfer the shear stress to red blood cells, resulting in red blood cells elongating. The extent of deformability - the sensitivity of the measurements - depends on the viscosity of the medium. In the current study we used polyvinylpyrrolidone (PVP) – phosphat buffered saline (PBS) solution (PVP: 360 kDa, Sigma-Aldrich Co. USA), PVP-PBS solution viscosity = 22.5 mPas, osmolality = 326 mOsmol/kg, pH = 7.1. During the measurements, 10 µl of blood was suspended in 2 ml of solution.

In the function of shear stress (0,5-30 Pa) the elongation index profile can be detected, where the higher elongation index reflect the well deformabled red blood cells.

Comparison of the results can be done by comparing EI with a selected shear stress value (EI at 3 Pa). These curves can be comparable by data parametrization by the Lineweaver-Burke analysis ($1/EI = SS_{1/2} / EI_{max} \times 1/SS + 1/EI_{max}$). We calculated the maximal Elongation index values (EI_{max}), and the shear stress belonging to the half of it ($SS_{1/2}$ [Pa]).

3.5.4. Red blood cell aggregation

The red blood cell aggregation was measured by Myrenne MA-1 Erythrocyte aggregometer (Myrenne GmbH, Germany), which technique is based on the light-transmittance method. For the measurements 20 µl blod

sample is required. Measurements can be performed at low shear rate (3 s^{-1}, M1 index) or at stasis (0 s^{-1}, M index). The instrument calculates the aggregation index values at the 5^{th} or 10^{th} second of the aggregation process. The aggregometer determine the aggregation index: M 5s, M1 5s, M 10s és M1 10s, where the higher aggregation index values reflect the enhanced red blood cell aggregation.

3.6. Histopathological examinations

At the end of the observed ischemia-reperfusion period samples were harvested from the left lateral lobe of the liver for histological examination. After fixing in 10% formaldehyde the samples were sent to the Department of Pathology, University of Debrecen, where the excised samples were embadded in paraffin and 4 μm serial sections were microtomed. For the analyzing of morphological changes caused by ischemia-reperfusion and inter-group differences Haematoxylin & eosin and PAS staining was used. For numerical evaluation of the severity of damage, the Suzuki's score system was applied. Experienced pathologist evaluated three different characteristic histological signs of ischemia-reperfusion injury (sinusoidal congestion, necrosis, vacuolisation) with 0 to 4 per experimental animal, depending on the severity of the difference.

3.7. Statistical analysis

For the statistical analysis SigmaStat software was applied (Systat software Inc., San Jose, California, USA).

All data were expressed as means± standard deviation (S.D.). ANOVA tests for intra-group comparisons (Bonferroni's/Dunn's methods) and Student t-test/Mann-Whitney rank sum test for inter-group differences were applied, depending on the normality of data distribution. $p<0.05$ value was considered statistically significant.

4. RESULTS

4.1. Respiratory rate, temperature

Regarding the respiratory rate there were no significant differences, however moderate elevation was observed in the Control and in the RIPC-24 groups during the reperfusion.

Comparing the experimental groups the same range of alterations was found in the different timepoints of the process regarding the rectal temperature.

Analyzing the values of the ischemic and non-ischemic liver lobe surface temperatures it can be determined that the non-ischemic liver lobe temperature increased in the Control and in the RIPC-1 groups at the end of the reperfusion (Control vs. Base p=0.049, RIPC-1 vs. Base p=0.012). In the RIPC-24 group the values elevated for the 60th minute of reperfusion (RIPC-24 vs. Base p=0.002).

Comparing the experimental groups the same range of alterations was found. For the 60th minute of reperfusion a significant difference was found between the Control and RIPC-24 groups (p=0.032 vs. Control). The temperature data of the ischemic liver was similar to the non-ischemic liver and the same tendency of changes was observed during the follow-up period. At the 30th minute of reperfusion the difference was significant in the Control group (p=0.015 vs. Non-ischemic liver).

4.2. Hemodynamic parameters

In the Control group the systolic blood pressure values were continuously decreased during the ischemia and at the 30th minute of reperfusion, then elevated again to the same level of the base value. The values dramatically increased at the 120th minute of reperfusion, which was siginificant compare to the base in the Control group (p=0.022 vs. Base). The systolic blood pressure level of both preconditioning group was lower compare to the Control group at

the beginning of the operation, however by the 120th minute of the reperfusion the values exceeded the systolic blood pressure level of the Control group.

The alterations of the heart rate were fluctuated moderately in the Control group, the values decreased significantly compare to the base (p=0.045 vs. Base). In the RIPC-1 group the values decreased at the 30th and 60th minute of the reperfusion, then elevated again by the 120th minute. The heart rates were approximately equal in the RIPC-24 group during the experimental protocol.

There were no statistically significant differences between the groups.

4.3. Microcirculation

Examining the BFU values of the ischemic part of the liver an identical pattern of alterations was demonstrated before the operation and at the end of the ischemia. In the RIPC-24 group the values were significantly decreased at the 30th minute of reperfusion (p=0.021 vs. Base; p=0.036 vs. Control). The BFU levels were moderately, but not significantly elevated in all groups by the 120th minute of reperfusion.

The BFU levels of the non-ischemic liver did not show relevant differences in the Control group. In the RIPC-1 group the BFU values were moderately elevated by the end of the ischemia, then continuously decreased for the end of the reperfusion period, without significant changes. The BFU parameters of the RIPC-24 group were continuously decreased till the 30th minute of the reperfusion, where the alteration was significant compared to the base and to the Control group as well (p=0.021 vs. Base; p=0.036 vs. Control).

The BFU level of the ischemic liver versus the non-ischemic lobe was significantly lower in the Control group at the 120th minute of the reperfusion (p=0.035 vs. Non-ischemic liver), and at the 60th minute of the reperfusion in the RIPC-1 group (p=0.043 vs. Non-ischemic liver).

The BFU parameters of the small intestine were increased at the 60th minute of ischemia in the Control and in the RIPC-24 groups, then decreased

during the reperfusion period, but there were no significant changes. At the 60th minute of the reperfusion the BFU was significantly lower in the RIPC-1 group compare to the Control group (p=0.038 vs. Control).

Comparing the BFU levels of the RIPC-1 and RIPC-24 groups, measured on the surface of the right kidney, the values were higher, but not significantly elevated, in the RIPC-1 group. In the RIPC-24 group the differences were significant at the 60th minute of ischemia and the 60th minute of reperfusion compared to the Control group (p=0.001 vs. Conrol I-60, p= 0.01 vs. Control R-60).

4.4. Laboratory measurements

4.4.1. Acid-base parameters

At the end of the examined reperfusion period the alterations of the pH values were mild in all groups. There were no significant inter- and intra-group differences.

At the end of the follow-up period lactate concentration was significantly increased in the Control and in the RIPC-1 groups compared to the base (Control: p=0.01; RIPC-1: p=0.009), however, there were no significant differences between the experimental groups. The relative values compared to the base were also determined. In the RIPC-1- group the data were higher compared to the RIPC-24 group (Control: p=0.016; RIPC-1: p=0.002; RIPC-24: p=0.029).

By the end of the reperfusion the *p*O2 values increased in all groups. The *p*CO2 parameters decreased in all experimental groups, where the changes were significant in the preconditioning groups (RIPC-1: p<0.001 vs. Base; RIPC-24: p<0.001 vs. Base). There were no significant differrences between the groups.

4.4.2. Hematological parameters

The red blood cell count and the hematocrit (Hct [%]) values showed a moderate elevation during the observed reperfusion period in the Control and in the RIPC-24 group. In the RIPC-1 group significantly lower hematocrit values expressed at the 30^{th} and 60^{th} minute of reperfusion (RIPC-1: p=0.021, p=0.039). Before the operation the differences were significant between the Control and the RIPC-1 groups (RIPC-1: p=0.049). However the level of the elevation was not significant, the hematocrit values continuously increased in the Control group during the observed period.

4.4.3. Red blood cell deformability

Analyzing the elongation index-shear stress curves it can be established that the red blood cell deformability improved in the RIPC-1 group, as the elongation index values were higher by the end of the reperfusion period.

In the RIPC-24 group the decrease of the elongation index at 3 Pa was significant at the 30^{th} (p=0.031 vs. Base) and 120^{th} (p=0.008 vs. Base) minute of reperfusion. The values were elevated at the 30^{th} minute of reperfusion in the RIPC-1 group (p=0.041 vs. Control) and at the 60^{th} minute of reperfusion in the RIPC-24 group (p=0.022) compare to the Control group.

Regarding the calculated parameters there were significant alteration in the ratio of the two calculated parameters ($EI_{max}/SS_{1/2}$) in the RIPC-24 group at the 30^{th} and 120^{th} minute of reperfusion (p=0.043 vs. Base; p=0.002 vs. Base).

4.4.4. Red blood cell aggregation

Four red blood cell aggregation index parameters (M 5s, M1 5s, M 10s and M1 10s) were determined by the Myrenne aggregometer.

In the Control group a moderate elevation was observed at the 30^{th} and 60^{th} minute of reperfusion, the aggregation index values decreased by the end of the reperfusion period.

At stasis the values decreased in both preconditioning groups during the reperfusion, but interestingly the results were controversial at low shear rate, where the aggregation index values were continuously elevated, especially in the RIPC-24 group.

The level of the elevation was significant in the RIPC-1 group at the 30th (M1 5s: $p=0.011$ vs. Base), 60th (M1 5s: $p<0.001$, M1 10s: $p=0.042$ vs. Base) and 120th (M1 5s: $p=0.002$, M1 10s: $p<0.001$ vs. Base) minute of reperfusion.

Aggregation index were significantly lower in the RIPC-24 group compared to the Control group before the operation (M 10s: $p=0.033$), at R-30 (M 10s: $p=0.008$), at R-60 (M5s: $p=0.031$). In the RIPC-1 group significant alterations were observed at the beginning of the operation (M1 5s: $p=0.05$), at R-60 (M 5s: $p=0.031$) and at R-120 (M1 5s $p=0.048$).

4.5. Histopathological examinations

In our current study the following histological alterations was observed after ischemia-reperfusion injury: sinusoidal congestion, pericentral desorganisation, necrosis in the pericentral region, hepatocyte dilatation, signs of inflammation, cytoplasm was more eosinophil and homogenous in the central zone. Quantifying the histological changes of the ischemic lobe of the liver a mild sinusoidal congestion, necrosis and in the 50% of the samples mild vacuolization was observed.

Examining the samples of the RIPC-1 group it can be determined that the deterioration of the tissue damage was more severe compare to the Control group, as the number of the necrotised hepatocytes was higher close to the sinusoids, furthermore congestion and inflammatory cells were described.

The samples of the RIPC-24 group showed similar histpathological changes, but the vacuolization was more serious, and the calculated Suzuki

score was also higher compare to the RIPC-1 and to the Control group (Control: 4.125±1.96; RIPC-1: 5.83±1.6; RIPC-24:6.13±2.12).

Based on the observed morphological changes, none of the applied preconditioning protocols could diminish the damage caused by ischemia-reperfusion. The anesthesia in the RIPC-24 group, and the effects of acute phase reactions that have started before the operation in both preconditioned groups could influence these alterations.

5. SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

1. We have developed a model where the effects of remote organ ischemic preconditioning prior to partial liver ischemia and reperfusion can be well investigated.

2. In the Control group the stability of the hemodinamic parameters decreased, we observed changes in the microcirculation, there was a significant increase in lactate concentration. The red blood cell deformability deteriorated, and red blood cell aggregation index values decreased at the end of the experiment after the initial moderate increase.

3. In the preconditioned groups, systolic blood pressure and heart rate fluctuations were lower than in the Control group, thus favoring the hemodynamic status of the experimental animals. We have shown that BFU values in the RIPC-1 group were higher till the 60^{th} minute of reperfusion in the monitoring of microcirculation. The lactate concentration increased moderately in the RIPC-24 group. Regarding the hemorheological parameters the red blood cell deformability was better on the 1 hour waiting period after preconditioning. The red blood cell aggregation index values were lower in both preconditioned groups compared to the Control group.

4. Analyzing histological results, it can be concluded that histopathological abnormalities were more pronounced in preconditioned groups. Based on the developed histological changes, it is not clear whether remote organ ischemic preconditioning has a beneficial effect on the prevention and reduction of histological damage.

The positive effects of remote organ ischemic preconditioning at two different timing were different for the different parameters studied. Further investigations are needed to clarify which protocol is more favorable and how long before the planned organ ischemia-reperfusion should be applied in case of the liver.

6. LIST OF PUBLICATIONS

UNIVERSITY of DEBRECEN

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H-4002 Egyetem tér 1, Debrecen
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Registry number: DEENK/28/2019.PL
Subject: PhD Publikációs Lista

Candidate: Zsuzsanna Magyar
Neptun ID: MHRDSL
Doctoral School: Doctoral School of Clinical Medicine
MTMT ID: 10054040

List of publications related to the dissertation

1. **Magyar, Z.**, Mester, A., Nadubinszky, G., Varga, G., Ghanem, S., Somogyi, V., Tánczos, B., Deák, Á., Bidiga, L., Mihai, O., Pető, K., Németh, N.: Beneficial effects of remote organ ischemic preconditioning on micro-rheological parameters during liver ischemia-reperfusion in the rat. *Clin. Hemorheol. Microcirc. 70* (2), 181-190, 2018.
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   Acta Cir. Bras. 33 (7), 597-608, 2018.
   DOI: http://dx.doi.org/10.1590/s0102-865020180070000005
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List of other publications

3. Mester, A., **Magyar, Z.**, Molnár, Á., Somogyi, V., Tánczos, B., Pető, K., Németh, N.: Age- and gender-related hemorheological alterations in intestinal ischemia-reperfusion in the rat.
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IF: 0.948

Total IF of journals (all publications): 11,588
Total IF of journals (publications related to the dissertation): 2,847

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

14 February, 2019

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

7. ACKNOWLEDGMENTS

I am grateful to my supervisor, Prof. Dr. Norbert Németh, head of Department, who introduced me into the research as a medical student, who created the opportunity to start my PhD work, and helped my research with his experience and endless support.

I thank Prof. Dr. Irén Mikó és Prof. Dr. István Furka for learning from them.

I would like to thank my collegue and friend Dr. Anita Mester for the unforgettable days spent together. I am grateful for her support, ideas and encouragement.

I am thankfull to Dr. Viktória Somogyi, assistant lecturer and to Bence Tánczos, PhD student, who didn't spare time and energy to give important help in the laboratory measurements.

I thank Dr. Katalin Pető, associate professor and Dr. Ádám Deák, assistant professor who gave me useful advices and help.

I would like to thank to Dr. Mihai Oltean (Gothenburg, Sweden), who helped me to learn the operative protocol.

I am grateful to Dr. László Bidiga (Department of Pathology, Faculty of Medicine) for the histological examinations.

Thanks to Rozália Gödényné for her help in the microsurgical operations.

I thank all the collegues of the Department of Operative Techniques and Surgical Research for the support.

I would like to thank to my Mother, who help me with love and support, who provided me a calm background for my studies. I am grateful for her pacience and encouragement.