THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D)

THE INVESTIGATION OF ISCHEMIA-REPERFUSION CHANGES ON SMALL INTESTINAL SEGMENTS AND SMALL INTESTINAL TRANSPLANTATION EXPERIMETNAL MODELS WITH THE APPLIANCE OF SURGICAL AND MICROSURGICAL TECHNIQUES

ENDRE BRÁTH M.D.

Supervisor: **PROFESSOR ISTVÁN FURKA M.D., Ph.D., D.Sc**

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

Debrecen, 2011.

1.INTRODUCTION

Still today, mesenterial ischemia is a disease with a high mortality rate. The small intestinal bowels can be injured due to mesenterial ischemic reperfusion; also for example due to small bowel transplantation, aortic and heart surgical procedures, hypovolemic shock, mesenterial infarction or neonatal necrotizing enterocolitis.

The mesenterial ischemic-reperfusion injuries are multifactoral, in which free radicals, nirtic oxide and leukocyte adhesion molecules play important roles. Also, molecules of the xanthin oxidase enyzmatic system, activated neutrophil leukocytes and endothel cells participate in the produciton of free radicals.

It is a known fact, that injuries envolving during *mesenterial ischemic-repefusion* pathmechanims induce apoptotic pathways depending on the extent of the inury. However, there are small available data exists about the changes duirng this process in the presence of *allupurinol* (xanthine-oxidase blocker), *vitamin E* (antioxidant) and *ischemic preconditioning* within the field of small intestinal transplantation.

The endogenic adaptation mechanims of ischcemic precconditioning enchances tolerance of oragns against the damege of ischemia-reperfusion. In several organs- liver, heart, skeletal muscle, small intestine, the protective effect of preconditioning was shown. However the clinical data is contreversial in regards, which is the most effective preconditioning technique in case of small intestine.

The success of transplantation is greatly influenced by the well developed blood supply of the organs. The complex process of ischemia-reperfusion initiate significant changes in several areas, also in the *microcirculation* of the intesine.

One of the important question of this reseach field is that how far the mircocirculatory disturbance of ischemia-reperfusion damages red blood cell deformability, filtrating ability, since its known that the red blood cells with rigid, damaged membrane passing ability is reduced.

While the previously raised questions can be investigated on large and small laboratory animals, the research of small intestine transplantation immunolgy is mainly conducted on smaller laboratory animals, such as rats, mice

Numerous genetically modified congenic, transgenic and knock out mouse strains provide an opportunity to clarify the processes. Different parts can be examined with widely available immunological, molecular biologic methods and with the application of poly-monoclonal antibodies.

The cost-benefit ratio is the best in case of small laboratory animals compared to large laboratory animals.

The disadvantage of the mice models that they require a special advanced *microsurgical experience* and practice due to the difficulty of the operative technique of microvascular anastomosis.

So it is important to develop a simple, quickly carried out experimental transplantation model, which might require basic microsurgical experience but can be easily learned and easy of attainment.

Therefore, it is a great importance to teach and to learn the basic microsurgical technique for the attainment and safely application of this microsurgical transplantation model.

In relation with intestinal transplantation, investigations of the previously mentioned and raised questions on 3 different laboratory animal species were done to answer the previously stated objectives, so later in time it can be applied in the clinical practice.

2. GOALS

I. During the investigations on mixed breed dogs:

1. To create a *double, isolated* jejunum segment with the possibilities to selectively compress the mesenterial branch of its blood supply for investigating the damage caused by mesenterial schemia-reperfusion.

2. To perform light microscopical investigation on the jejunum-segment model, especially to investigate the time course of apoptotic process in the mucosal wall of the small intestine during a 30 minutes ischemia-reperfusion.

3. To determine on the jejunum-segment model, which preconditional time periods are effective. Does preconditioning influence the apoptosis of the mucosa during ischemia-reperfusion?

4. To prove the hypothesized protective characteristics of Vitamin E and allopurinol. For this reason, do Vitamin E and allopurinol affect the apoptotic cascade in the mucosa of the bowel during ischemia-reperfusion?

5. To investigate the change in red blood cell deformability with hemorheological methods during ischemia-reperfusion after the total clamping of superior mesenteric artery.

II. During the investigation on Outbred rats.

6. To demonstrate the changes of certain hemorheological parameters in the microcirculation with newer measuring methods after atraumatic clamping of the superior mesenteric artery during mesenterial ischemia-reperfusion.

III. During the investigations on Inbred mice

7. To execute a small bowel transplantation model without a vascular anastomosis, so called "avascular" with microsurgical methods.

8. To examine the viability of the avascular small bowel transplants with histological methods.

9. To develop further the basic microsurgical training for transplantation research.

3. INVESTIGATION OF THE DAMAGES CAUSED BY MESENTERIAL ISCHEMIA-REPERFUSION ON JEJUNUM SEGMENTS.

3.1. MATERIAL AND METHOD

3.1.1. Experimental animals, anesthesia

The experiments were conducted under the XXVIII. Law of year 1998, "Animal protection and welfare" and with the permission issued by the University of Debrecen Committee of Animal Research (UDCAR) (Permission number: 56/1997/ÁTEB, 24/1998/ÁTEB, 22/1999/ÁTEB).

The experiments were performed on 51 mixed dogs, weighting between 18-24 kg (23.4±2.63 kg).

Anesthesia was induced and maintained by combined administration of SBH-Ketamin (10% ketaminum hydrochloricum, 10 mg/kg, i.m.) and Primazin (2% xilazinum hydrochloricum, 1 mg/kg, i.m.).

In all the animals included in the experiments, we created our elaborated double jejunum segment model, and then examined the damages caused by ischemia-reperfusion and the protective factors against it.

3.1.2. Operative technique for double-isolated jejunum segment

After performing a median laparatomy, the small intestine was isolated, and then two 15 cm long jejunum parts were selected with its blood supplying vessels. Afterwards, the bowel wall was transected at three sites, thus only the mesenteric vessel would assure the blood supply to the segment.

Consecutively, three end-to-end anastomosis were performed with the use of PDS (polydioxanone) 3/0 suturing material at both ends and in the middle portion of the intestinal loop to maintain the continuity of the gastrointestinal tract. By this two equal 15 cm long jejunum segments were created, which blood supply was assured only by its mesenterial arterial branches because they were not be able receive blood circulation from the neighboring segment. Investigations were performed on one segment without vessel compression, the control group, and on the other segment with occlusion of its supplying vessels using atraumatic compression where we waned to carry out the ischemia-reperfusion investigations.

In the following, 30 minutes ischemia was induced by occluding the vessels of one segment (ischemic segment) using an atraumatic vascular clip for further differentiation, comparative examinations.

3.1.3. Experimental groups in the investigation of the course of reperfusional damage

The 24 operated animals weighting 23.4±2.63 kg were divided into 8 groups, 3-3 animals in each. In all groups, the supplying arteries of the jejunum segments applied for ischemia-reperfusional investigations, were atraumatically compressed for 30 minutes.

After releasing the compression of the vessels, reperfusional investigating periods with different duration followed with 30 minute, 1, 2, 4, 6, 8, 12 and 24 hours of the reperfusion time. By the end of reperfusion periods, biopsies were taken from the *ischemia-reperfusion* jejunum segment and from the *control* jejunum segment without arterial compression.

3.1.4. Experimental groups in the investigation of ischemic reperfusion with different durations

The 15 operated animals weighting 22±1.38 kg were divided into 5 groups, 3-3 animals each.

In the *intact control group*, interventions were not performed on the double, isolated jejunum-segment.

In the *compressed/ischemia-reperfusion control group*, the supplying vessels of the double, isolated jejunum-segment were atraumatically compressed after a 10 minutes stabilization phase to perform I/R investigations on them, and then biopsies were taken from the compressed and the intact control group after 4 hours of reperfusion.

In the *preconditioning group I*, prior to the 30-minute ischemia preconditioning protocol was used that included three times performed 5-minute ischemic period with 1-minute intermittent reperfusion phases and was followed by 10 minutes of releasing-stabilizing phase. Thirty minutes of ischemia was followed by 4 hours of reperfusion.

In the *preconditioning group II,* the supplying vessels of the examined segment were preconditioned for 3x3 minutes and was followed by 10 minutes releasing-stabilizing phase. Similarly to the previous group, 30 minutes of ischemia was followed by 4 hours of reperfusion.

In the *preconditioning group III,* the supplying vessels of the examined segment were preconditioned for 15 minutes (15 minutes of ischemia without any reperfusion period), and was followed by 10 minutes releasing-stabilizing phase. Similarly to the previous group, 30 minutes of ischemia was followed by 4 hours of reperfusion.

At the 4th hour of the reperfusion tissue samples were taken from the ischemic and control jejunum-segments for light microscopy and immunohistochemical staining.

3.1.5. Experimental groups in the investigation of preconditioning, and the pretreatment/preliminary treatment with allopurinol and Vitamin E

The 12 experimental animals with average weight 23±1.26 kg were divided into 4 groups, 3-3-animals each. The previously described double, isolated jejunum-segments were created in the experimental groups.

In the *compressed/ischemic control group* the supplying vessels of the examined segment were atraumatically clamped for 30 minutes after the stabilization phase of the operation, and then was followed by 4 hours of reperfusion.

In the *preconditioning group I,* the supplying vessels of the examined segment were preconditioned through 3x5 minutes (1 minute reperfusion period between 5 minutes of ischemic period), then was followed by 10 minutes releasing-stabilizing phase. Afterwards, 30 minutes of ischemia was followed by 4 hours of reperfusion.

In the *allopurinol pre-treated ischemia-reperfusion group*, prior to the 30 minutes ischemia 50-mg/kg allopurinol (125 mg in 20 ml 0.9 % saline solution) was infused into the external jugular vein. Then the ischemia-reperfusion was carried out as in IR group.

In the *vitamin E pre-treated ischemia-reperfusion group,* 25 mg/kg vitamin E was given intramuscularly prior to operation, and then 4 hours of reperfusion followed after 30 minutes compression of the mesenterial vessels.

At the 4th hour of the reperfusion tissue samples were taken from the ischemic and control jejunum-segments for light microscopy and immunohistochemical staining.

3.1.6. Morphological investigation methods

3.1.6.1. Macroscopic observations

At the end of the reperfusion periods prior to the biopsies macroscopic observation of the abdominal cavity of the experimental animal and the region of the anastomosis were carried out. The lumen of the intestine was opened, its content was observed, and then total cross sections of the bowel wall were sampled for microscopical investigations from preconditioning groups, the ischemic group and the intact control group without compression.

3.1.6.2. Microscopic investigations

During the preparation of the jejunum-segment, histological samples were taken from the bowel wall prior to creating the anastomosis for comparative investigations.

3.1.6.2.1. Conventional histological techniques

Biopsies of the intestinal wall were fixed in 10 % formalin, dehydrated in a graded series of alcohol, embedded in paraffin, microtomed in 5 μm step sections, and stained with haematoxilin and eosin (H&E).

3.1.6.2.2. Immunohistochemical technique to detect apoptosis

Apoptosis was detected by using Apoptag Kit ((in situ apoptosis detection kit, Oncor, Biomarker Ltd.). The apoptotic cells were identified with TUNEL (terminal deoxy-nucleotidyl-transferase-mediated, dUTP nick end-labeling) technique. The investigation followed the protocol of the manufacturer. During apoptosis the activated endonucleases in the nucleus could cause the breaking of one of the DNA chain (nick). Terminal deoxynucleotidyl transferase dUTP nick end labeling is a method for detecting DNA fragmentation by labeling the 3'OH terminal end of nucleic acids with digoxin.

3.1.6.2.3. Counting the apoptotic cells to determine the time course of the reperfusional damage

In the first phase of the investigation, when we determined the optimal reperfusion time, the comparison between groups were carried out based on simple methodology - having regard to the large amount of biopsies. TUNEL positive cells were counted under ocular micrometer at 40X magnification in 10-10 visual fields. Statistical analyses were done with application of the SPSS 10.0 software, and with the help of ANOVA on ranks (Dunn's method) test ($p<0.05$).

3.1.6.2.4. Calculation of apoptotic index in the investigation of ischemic preconditioning with different time periods, and pretreatment with allopurinol and vitamin E

The apoptotic index was calculated for a more accurate comparison: the number of positively stained nuclei was divided with the number of villi. Fifty villi were examined per section. Statistical analyses were done with application of the SPSS 10.0 software, and with the help of Student's t-test.

3.2. RESULTS

3.2.1. Macroscopic observations

During the experiment there was neither mortality nor any complication in the abdominal cavity and in the operating region. All the intestinal anastomosis functioned well and the intestinal sutures were intact.

During biopsy sampling, after opening the bowel wall we could see that there was a small quantity of debris adhered on the intestinal mucosal surface at the 30th minute, 1st and 2nd hour of reperfusion. Similarly, small quantity of debris was found at the 12^{th} and 24^{th} hours of reperfusion.

However, at the 4th, 6th and 8th hours of reperfusion the intestinal lumen was full with *white cell debris*, which was strongly adhering to the intestine wall. It seemed like the mucosa detached from its surface. This layer was not removed.

3.2.2. Microscopic investigations

3.2.2.1. Conventional histological techniques

H&E staining demonstrated minimal cellular debris on the intestinal mucosal surface at the *30th minute, 1st and 2nd hour* of reperfusion. In several sections, subepithelial spaces were observed in the apical region of villi without serious mucosal damage. Numerous granulocytes, mostly lymphocytes were found in the apical region. The structure of the intestinal wall remained intact.

In the tissue samples taken at the *4th, 6th and 8th hours of the reperfusion* the mucosa was intact, but several mucosa were characterized by development of subepithelial Gruenhagen's space, usually at the apex of the villus with moderate lifting. In certain villi extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria was also observed. Mainly lymphocytes were seen. The deeper layers of the intestinal wall were well organized, damage was not found.

In biopsy materials taken at the 8th, 12th and 24th hours of reperfusion there was no damage in the deeper structure of the intestinal wall. In the control samples mucosa was normal without inflammatory cells.

3.2.2.2. Immunohistochemical investigations observing the time course of reperfusional injury

At the end of the *1st and 2nd hours* of the reperfusion apoptotic cells were observed in great number, especially in basal and apical areas.

The apoptotic activity increased gradually in the investigated samples reaching its peak after 4-6 hours of reperfusion. In the following, the number of TUNEL positive apoptotic cells was greatly decreased in the 12- and 24 hours samples. The number was similar with the control specimens. The results were summed in Table I.

Table I. - The number and localization of apoptotic cells in the function of reperfusion time

Duration of the reperfusion	The number of the TUNEL positive apoptotic cells /visual field at 40x magnification	Localization of the apoptotic cells in the intestinal villi
0 minute (Control)	0.7 ± 0.57	basal and apical region
30 minutes	2.96 ± 0.61 *	basal and apical region
1 hour	3 ± 0.74 *	basal and apical region
2 hours	6 ± 1.17 *	apical region
4 hours	14.2 ± 1.31 *	at the tip of the villi, basal region and in the crypts
6 hours	16.3 ± 1.05 *	at the tip of the villi, basal region and in the crypts
8 hours	3.83 ± 0.79 *	basal and apical region
12 hours	0.8 ± 0.61	basal and apical region
24 hours	0.83 ± 0.59	basal and apical region

* $p<0.05$ vs 0 minutes (Control), one way ANOVA on ranks (Dunn's method)

3.2.2.3. Conventional histologic results in the investigations of ischemic preconditioning, and the pretreatment with allopurinol and Vitamin E with different time periods.

In all biopsies, *stained with H&E had similar results*; practically there was no difference.

Within the intestinal lumen, significant amount of cellular debris were observed on the surface of the mucosa in every 3x5, 3x3, and 1x15 minutes samples of the preconditioned or pretreated with allopurinol and Vitamin E groups. Lobulated mucosa and tunica propria were noticed with high lymphocytic count. Extensive subepithelial space was seen frequently with mucosa lift-off in the apical region the villi, too. The deeper layers of the bowel wall were intact and damage was not observed.

3.2.2.4. Immunohistochemical results in the investigation of ischemic preconditioning, pretreatment with allopurinol and Vitamin E with different time periods

In the *Compressed control* groups, the value of apoptotic index was 2.76±1.19.

Only the *3x5 minutes preconditioned group*, among the different time periods, showed *significantly elevated* apoptotic index *compared to the compressed control group*, which was 6.75±1.2. For this reason, we had chosen 3x5 minutes from the different ischemic preconditioned methods for the comparison with a well-known protective drug against ischemic reperfusion injury.

The apoptotic index was 11.3±3.1 in the group *pretreated with allopurinol*. Large amount of cellular debris was visible on the surface of the mucosa. The value of apoptotic index were *significantly elevated* compared to *Compressed control* (p=0,002) and preconditioned groups.

The highest apoptotic index was 12.5±3.25 after the use of *Vitamin E*, which *was significant* compared to the C*ompressed control* and P*reconditioned I. groups* apoptotic cells were observed around the apical and basal regions of the villi. Epithelial and granulocytes showed TUNEL positivity within the mucosa. The lumen was covered with a thick cellular debris layer (Table II.)

Table II. Apoptotic index values in relation to ischemic preconditioning, pretreatment with allopurinol and Vitamin E in different time periods

Experimental groups	Compressed control	Preconditioning I. group (3x5 minutes)	Preconditioning II. group (3x3 minutes)	Preconditioning III. group (1x15 minutes)	Pretreatment with Allopurinol group	Pretreatment with Vitamin E group
Apoptotic index: TUNEL positive nuclei / 50 villi	**2,76±1,19**	**6,75±1,2***	**3,85±1,25**	**2,81±1,3**	**11,2±3,1$^{\#\ \wedge}$**	**12,2±3,25$^{+\wedge}$**

*p=0,014, #p=0,005,+p=0,002 vs Compresed control; ^p<0,05 vs Preconditioning I. group, Student's t-test

3.3. DISCUSSION

The injuries caused by the ischemia-reperfusion originate from many factors, such as reactive oxygen intermediers, leukocytes adhesion molecules, nitrogen oxide, but not all factors are known in this process.

During ischemic-reperfusion the main source of the free radicals next to the activated neutrophil granulocytes, is the xanthine-oxidase/dehydrogenase system. The intestine contains

the highest quantity of xanthine-dehydrogenase enzyme, which under ischemic conditions converts into xanthie-oxidase. The process is the fastest in intestinal tissue. During anoxia, the ATP molecule is dephosphorilating in the cell, thus the concentration of AMP is increasing. Therefore in the cells suffering from hypoxia xanthine-oxidase and of its substrates are present. During reperfusion-reoxygenation the other substrate, the molecular oxygen appears and the xanthine-oxidase starts to reduce the molecular oxygen into superoxide radical and hydrogen peroxide.

Granger et al. demonstrated that the xanthine-oxidase presented in the highest activity in the apical region of the villi, therefore the damage caused by the free radicals are mire intensive in this area. These finding may explain, why the histological samples showed so intensive damage in the mucous membrane and why the number of apoptotic cells at the 4^{th} and 6^{th} hour of reperfusion was so high.

There are no unequivocal articles concerning *the time course of apoptosis* caused by mesenterial ischemia, therefore we examined this during our experiment

It is well known that apoptosis requires active protein synthesis, which needs time, therefore this could be the explanation why the umber of apoptotic cells was low in number at the 30^{th} minute, 1^{st} and 2^{nd} hours of reperfusion.

The highest apoptotic cell count was between the *4^{th} and 6^{th} hours of reperfusion,* therefore in our model 30 minutes ischemia, 4^{th} and 6^{th} hours of reperfusion was *the most suitable time period for investigation of apoptosis.*

The number of apoptotic cells was low at the 8^{th}, 12^{th} and 24^{th} hours of the reperfusion. It is suggested that the process finished, thus further investigations over these periods of reperfusion time are not appropriate.

Also, the clinical data is not unified in the application of *preconditional methods* with different time periods. Yellon et al. described that in different animal species there were different molecular mechanisms predominate during the ischemic preconditioning. They found difference during the preconditioning of the heart in rats, rabbits, dog, and pigs.

In our experiments, we wanted to examine the effectiveness of different preconditioning protocols and their effect on apoptosis. The results show that *the apoptotic cell count increased in the bowel wall due to effect of preconditioning*. Especially the 3 x 5 minutes method increased significantly the number of TUNEL positive cells compared to the control group. The 3 x 3 minutes method also increased the number of apoptotic cells, while the 1 x 15 minutes preconditioning did not cause any change in our experimental model.

Our prior assumption was, that preconditioning would decrease the rate of apoptosis emerging from ischemic reperfusion because the ischemic preconditioning provides protection against the damages caused by ischemic reperfusion. However, our results contradicted this. Explanation might be that in our mixed dog model the used preconditioning methods would not be able preventing the damages caused by 30 minutes ischemia with 4 hours reperfusion.

Presumably, the higher intracellular energy level of the preconditional cells contributed to the possibility of apoptotic cells death.

Ferencz et al. found similar results in mixed dogs using 4 x 5 minutes of ischemic preconditioning. They detected DNA damage with TUNEL technique. In the preconditioned group, elevated levels were found compared to the control group. They attributed a key role to NF- κB in the transduction pathways and in the mechanism of preconditioning used to counteract ischemic reperfusion, next to the well-known xanthine-oxidase, activation of neutrophil granulocytes.

During the application of *allopurinol pretreatment*, we obtained result opposite to our assumption, similar to out preconditional experience, so it did not decrease, but t*he number of*

apoptotic cells increase in the intestinal mucosa. This could suggest that several factors may play a role in the development.

During ischemic reperfusion the oxidative stress could be decreased with allopurinol pretreatment, which contributes to cell survival by reducing the level of reactive oxygen radicals. The intracellular calcium accumulation, the usage of ATP may moderately be decreased. Beside the increase in number of surviving cell number, it might facilitate apoptotic cell death against necrosis. Shah et al confirmed, that ischemia- reperfusion cause apoptosis in the intestines but also concluded that after ischemia- reperfusion both apoptosis and necrosis can develop. He considers apoptosis the primary cause of cell death as opposed to necrosis.

Nicotera et al. considers the ATP level as a determinative factor in reference to the cells diminishing either in an apoptotic or necrotic way. If the intracellular ATP level was low the signal stimulating apoptosis can cause necrosis. The intracellular energy level influences what types of cell death would be initiated.

McKoney et al determined the conditions for apoptosis/necrosis in the following. *Apoptosis*: ATP shortage of 25-50%, increased level of Ca^{2+} 200-400nM, moderate free radical formation, activation of caspase system is necessary for the process. *Necrosis*: ATP shortage of 70-100%, increased level of Ca^{2+}>1μM, large amount of free radical formation, Bcl-2 activation free radical production, Bcl-2 inhibition, no activation of caspase system were required/necessary for the process.

Shue et al. considers that *vitamin E* blocks the activation of NF- κB. The participation of NF-κB had been demonstrated in several processes, for example in ischemia-reperfusion, in apoptosis, and in the intestinal reperfusion cascade, a systemic inflammatory response.

Nichols et al. found in relation with mesenterial ischemia, that if we block the activation of NF- κB, then the level of apoptosis increase in the mucosa after reperfusion.

The result of our experimental model gave explanation to the presumption of the clinical data, in which blocking the activation of Vitamin E and NF- κB would enhance the apoptosis in the mucosa of the bowel wall during damage caused by ischemia-reperfusion.

Summarizing our results we concluded that if the ischemic insult was milder or certain protective factors (preconditioning, allopurinol, Vitamin E) were present, the majority of ischemic cell death was by apoptosis. Inflammation and tissue damage appeared to be a lesser extent in the tissue, providing favorable conditions for tissue regeneration.

4. INVESTIGATION OF HEMATOLOGICAL AND HEMORHEOLOGICAL CHANGE CAUSE BY MESENTERIAL ISCHEMIA-REPERFUSION AFTER COMPRESSING THE SUPERIOR MESENTERIC ARTERY - II. EXPERIMENTAL MODEL

To our knowledge, there are small available data on the damage caused by ischemia-reperfusion and deformability changes of red blood cells in the clinical, therefore one of our objective was to investigate this issues.

4.1.MATERIAL AND METHOD

4.1.1.Experimental animals, anesthesia

The experiments were performed on 10 mixed dogs, weighting between 22-25 kg (23,4±2,63kg) Permission number: 22/1999/ÁTEB

The anesthesia of the experimental animals was carried out as it was previously described in section 3.1.1.

4.1.2. Operation protocol, experimental groups

1. *Ischemia-Reperfusion [I/R] group* (n=5): after a median laparatomy, we isolated the superior mesenteric artery, applied 30 minutes of depression, then the abdominal wall was closed in 3 layers.

2.*Sham Operated Control [SC] group* (n=5): after a median laparatomy, we closed the abdomen with 3 layers.

4.1.3. Laboratory investigation, statistical analysis

The laboratory investigations were done one day before the operation (base sample), after the operation 30 minutes, 1, 2, 4, and 6 hours as well as on the 1st, 2nd, 3rd, 5th, and 7th postoperative days. The statistical analysis was carried out with the help of Repeated Measuers, T-test and Mann-Whitney type Rank –Test ($p<0.05$).

4.1.3.1. Measurement of red blood cell deformability with filtrometer

The samples were taken from peripheral blood, small saphenous vein into vacutainers with Na-heparin (143 IU, 7 ml, BD Vacutainer® Belliver Industrial Estate, UK).

After 10 minutes of centrifuge, removal of the buffy coat and the plasma of the blood samples anticoagulated with Na-heparin the samples were diluted with phosphate puffer (PBS), and then before removing the supernatant they were centrifuged again. The red blood cell suspension diluted with PBS in 1:1 proportion, then we determined the hematocrit value with Janetzky-capillary centrifuge (5 mins). According to the obtained hematocrit, we further diluted the samples with PBS to 5% cell suspension hematocrit required for the measurement.

To measure the filterability of the red blood cell suspension Carat FT-1 filtrometer was used (Carat Ltd., Hungary) based on St. George's filtrometer technique. Measurements were carried out within two hours after blood taking.

The measurements were in a controlled 22±1 ^{o}C environment. The 5% erythrocyte suspension was flown through a polycarbonate filter with a mean pore diameter of 5 μm (Nuclepore, Whatman Inc.) at a constant 4 cm H_2O flow pressure. The filtration rate of PBS solution, which during the calibration was passed through was the control. The software calculated the filtration rate from the signals of four pair of lights sources and photodetectors, and then set the initial relative filtration rate (IRFR) and the relative cell transit time (RCTT), based on the entered suspension-hematocrit value. Both of the parameters are without dimension. The RCTT values are inversely proportional to the red blood cell deformability: the transit time is prolonged because the more difficult deformation of red blood cells caused slower flow through the filter.

4.1.3.2. Hematological investigations

The following quantitative and qualitative hematological parameters were determined from the blood samples in K3-EDTA anticoagulant containing Vacutainer (7,5%, 2 ml, BD Vacutainer® Belliver Industrial Estate, UK) with Sysmex F-800 type microcell counter (TOA Medical Electronics Co., Ltd., Japan): red blood cell number (RBC [x106/μl]), white blood cell number (Fvs [G/l]), haematocrit (Htc [%]), haemoglobin concentration (Hgb [g/dl]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin (MCH [pg]), mean corpuscular hemoglobin concentration, (MCHC [g/dl]), red cell distribution width (RDW-CV [%]), platelet count (Thr [x103/μl]), mean platelet volume (MPV [fl]).

4.2. RESULTS

4.2.1. Changes of red blood cell deformability

The RCTT did not show significant difference in the first hour between the *Ischemia Reperfusion group* and *Sham Operated group*.

However, in the 2nd hour of reperfusion there was a slight increase RCTT, but compared to the 1st hour was significant in the *Ischemia-Reperfusion group*. The most remarkable decay of red blood cell deformity was shown on the 3rd postoperative day with the significant increase of RCTT value not only in the *Sham Operated group*, but *also in Ischemia-Reperfusion group* compared to the values of 2nd postoperative day. The RCTT value was still significantly elevated on the 5th postoperative day, but it started to decrease to its baseline value on the 7th postoperative day.

4.2.2. Changes of hematological parameters

The *white blood cell count* showed gradual increase in the *Ischemia-Reperfusion group* within the first 4 hours of reperfusion, which reached its peak at the 6th hour. The elevated WBC was significant ($p<0.001$) compared to the values of both the initial and the control. We still detected significant increased white blood cell count on the 1st, 2nd, and 3rd postoperative day, which started to decline on the 5th day. Also on the 1st and 2nd postoperative day of the *Sham Operated group* the white blood cell count was significantly increased compared to the baseline values, however it stayed under the value of the ischemia reperfusion group.

The *red blood cell count* did not show significant change during the entire period, but based on the change of the average value it can be said that the red blood cell count increased 7.5% on the 1st postoperative day in the *Ischemia-Reperfusion group*, then it showed a decrease during the first postoperative week: on the 7th postoperative day the red blood cell count was 95.47% of the initial value (preoperative baseline value). The *hemoglobin level* and the *hematocrit value* changed parallelly, but the difference was a mild degree of hemoconcentration on the 1st postoperative day, which indicated only 3.81% increase in hematocrit while it decreased under 88.22% of the initial value on the 7th postoperative day.

In the *Ischemia-Reperfusion group*, the mean corpuscular volume, the mean corpuscular hemoglobin, and the mean corpuscular hemoglobin concentration values changed slightly on the 3rd and 7th postoperative day due to the change in the value of hemoglobin and hematocrit.

The *platelet count* barely changed during the entire investigating period in the *Ischemia-Reperfusion group* due to the variability of individual values it showed a large standard deviation. We measured 90.12% of the average initial value of the platelet count on the first postoperative day, which showed a slight increase between the 2nd and 7th postoperative days. We found 15.44% increase compared to baseline value on the 7th postoperative day. All of the above was accompanied by a mild change in the *mean thrmobocyte volume* (1st postoperative day: 3.63% increase, 7th postoperative day: 4.3% increase). Such changes were not observed in the control group.

4.3. DISCUSSION

It is well known from clinical practice that the possible complications of early postoperative period could be the reason for the development of several experimental models, in which the significant decay of several hemostaseologic, hematologic, and hemorheologic parameters on the first 3 postoperative days were proven with the investigation of ischemia-reperfusion with different time periods in several organs.

The ischemia with different time period and with different tissue extension depending of the type of the tissue and the following reperfusion can impair the red blood cell deformability to a varying degree.

The events of early postoperative days may differ from the early period of reperfusion. Directly after ischemia mainly metabolic changes and free radicals, which were released due to the xanthine-oxidase system activity during the reperfusion of the ischemic tissue, influenced the deformability of red blood cells. Depending on the tissue damage, the activation of polymorphonuclear leukocytes and the effect of free radicals from the their activation, resulted the decrease of filterability of red blood cell suspension in the first couple of postoperative days (dominant in 3rd postoperative day).

According to the clinical data, after releasing the vessel even 10 minutes or 15 minutes ischemia immediately deteriorated significantly the deformability of red blood cells and increases the aggregation capability of red blood cells. Data obtained from the investigation of different organs and organs systems, only comparable with each other on a theoretical level, since ischemic tolerance was significantly tissue type dependent, and the small intestine was the most sensitive tissue to damages caused by ischemia-reperfusion.

Based on our result, the *investigation of red blood cell deformability*, especially measuring the RCTT might provide *information on the condition of the small intestine after the operation,* which requires the compression of the superior mesenteric artery on the 1-3rd postoperative days in case of the possibility of complications. It may also be good method to demonstrate the damage occurring during ischemia-reperfusion and the early indication of the involvement of small intestine.

5. CHANGES OF HEMORHEOLOGICAL AND HEMATOLOGICAL PARAMETERS IN LOCAL AND SYSTEMIC CIRCULATION DURING ISCHEMIC REPERFUSION COURSE IN RATS - III. EXPERIMENTAL MODEL

Our objective by continuing our hemorheological experiments during the mesenterial ischemia-reperfusion was to investigate the emerging changes in the local circulation next to the systemic circulation with newer methods.

5.1. MATERIAL AND METHOD

5.1.1. Experimental animals, anesthesia

The experiments were performed on 10 CD outbred rats weighting 360± 46.07g (Permission number: 6/2006. DEMÁB).

After subcutaneously administered Atropin premedication (1% atropinum sulfuricum, 0,04 mg/bwkg, Paines&Byrne, Ltd.), the anasthesia was induced and maintained using Nembutallal (Na-pentobarbital, 35 mg/bwkg, Phylaxia-Sanofi, Ltd), intraperitonally.

5.1.2. Operative Technique and Experimental Groups

1. *Ischemia-Reperfusion (I/R) group*: median laparatomy was performed, and under operating microscope (Leica Wild M650, LEICA Ltd., Germany) gentle preparation of the superior mesenterial artery was carried out, then it was clipped atraumatically for 30 min. After removing the clip, 60 min of reperfusion was observed before extermination.

2. *Sham Operated (SO) group:* after a median laparatomy the preparation of the superior mesenteric artery was carried out and clips were not applied.

5.1.3. Laboratory investigation, statistical analysis

Blood sample were taken the portal vein and from the caudal vein. During the sampling 0.2 ml of blood was obtained with a 1 ml syringe. Anticoagulation was carried out with the help of K3-EDTA (a.5mg/ml) in the conus of the syringe.

After the preparation of the superior mesenteric artery in the *Ischemia-Reperfusion (I/R) group* the first sample was taken. In the following we took sample 1 min before and after clip removal (R+1; R+1), and at the 15^{th} (R + 15), 30^{th} (R + 30), and 60^{th} (R + 60) minutes of the reperfusion.

In *Sham Operated (SO) group*, samples were obtained at the similar time points with the *Ischemia-Reperfusion (*I/R) *group*. The sampling protocol was identical in both experimental groups.

ANOVA on ranks test and Mann- Whitney Rank sum test were used for statistical analysis. $P < 0.05$ was regarded as statistically significant.

5.1.3.1. Investigation of red blood cell deformability with ektacytometer

Red blood cell deformability was determined by RheoScan D-200 slit-flow ektacytometer (Sewon Meditech Inc., South Korea).

This instrument combines the laser diffraction technique with the slit flow rheometry. Based on the protocol of the ektacytometer, the sample was taken into a special container with a capillary (d=216micm). Afterwards the automat places the sample under vacuum pressure, thus the red blood cells elongate in situ in a high viscosity medium. The light of laser diodes pass through the microslit, capillary, so we get a picture, sample from the shape of the cell that is in the slit. From this it calculates, with a help of software, the elongation index, which is equal to (L-W)/(L+W) ratio, where L is the length and W is the width of the deformed cell, and next to a constant shear stress.

With EI-SS curves Lineweaver-Burke type analyses were carried out in order to determine maximal elongation index (EI_{max}) and the shear stress at half maximal deformation ($SS_{1/2}$). The maximal calculated elongation index is a value, which depends on the cell molecular and structural parameters, reaches the maximal extensibility at a given shear rate. So in a set shear rate range (SS: 0-20 Pa), with the analysis of the laser diffractogram measures the red blood cell deformability in PVP (Polyvinylpyrrolidon) a high viscosity medium. The high viscosity medium blocks the displacement of the cells in the capillary, however the forces acting on the cells are able to deform them. In general we can state, that Red blood cell deformability is worsening with decreasing of EI_{max} and/or increasing of $SS_{1/2}$.

5.1.3.2. Measurement of red blood cell aggregation

As a supplementary test, we determined the red blood cell aggregation at the last minute of 30 minutes ischemia (R – 1) before the removal of the clips and the end of reperfusion (R + 60). We performed the measurement with the help of Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany). The instrument operates with the optic aggrometric theory, which is based on the measuring intensity of light passing through or reflecting back from the blood principle.

5.1.3.3. Hematological parameters

The laboratory measurements were carried out under standard conditions. A Sysmex F-800 microcell counter (TOA Medical Electronics Corp., Ltd., Japan) was used to determine qualitative and quantitative parameters. The method of measurements and the measured parameters are same as the previously described in section 4.1.3.2.

5.2. RESULTS

5.2.1. Red blood cell deformability

There was no significant difference in the maximal elongation index (EI_{max}) values in the caval and portal vein samples of both groups during the examined periods. EI_{max} values ranged between 0.553 and 0.560.

On the other hand, shear stress values at half maximal deformation ($SS_{1/2}$ [Pa]) showed impressive changes during the last 30 min of reperfusion.

In the *portal vein* samples of *Ischemia-Reperfusion (I/R) group*, we could detect significant elevation of $SS_{1/2}$ just before starting the reperfusion (2.25 ± 0.44 Pa, p=0.014) and after removing the clip (2.30 ± 0.46 Pa) compared with the base (1.87 ± 0.25 Pa, p=0.009). $SS_{1/2}$ reached the highest values by the 30th min of the reperfusion (2.43 ± 0.65 Pa) and slightly decreased at the 60th min (2.38 ± 0.56 Pa), both are being significantly elevated versus base and compared with the *Sham Operated group* (Sham R-30: 1.71 ± 0.13, $p < 0.05$ and 1.68 ± 0.20 Pa, $p < 0.05$, respectively).

In the *caval vein samples of I/R group*, an elevation of $SS_{1/2}$ was observed at the 30th and 60th min of the reperfusion (2.14 ± 0.54 and 2.27 ± 0.50 Pa) versus base (1.80 ± 0.26 Pa) and versus *Sham Operated group* (1.55 ± 0.13 and 1.62 ± 0.20 Pa, $P < 0.001$, respectively).

5.2.2. Red blood cell aggregation

Red blood cell aggregation values were higher in portal blood by the end of ischemia (R-1) and by the end of reperfusion period (R + 60) compared with the caval venous blood. At these time periods the portal per caval mean ratio of erythrocyte aggregation index values were 1.86 (portal: 6.55 ± 0.37 vs caval: 3.51 ± 0.62) and 2.36 (portal: 4.06 ± 0.78 vs caval: 1.72 ± 0.16) suggesting that erythrocyte aggregation is more intensive in portal venous blood.

5.2.3. Hematological parameters

From the measured hematological sample, we present only the significantly changed results.

Change in hematocrit: Hematocrit (Hct [%]) of caval venous samples of *Sham Operated group* slightly decreased during the experimental period, while in *Ischemia-Reperfusion group* a significant elevation ($p<0.05$) was observed, reaching its peak in the first min of the reperfusion. In portal venous blood samples the tendency was similar. There was no significant difference between portal and caval vein data at any time.

Change in mean corpuscular volume: In *Ischemia-Reperfusion group* mean corpuscular volume values were continuously and significantly elevated during the reperfusion both in caval and portal venous blood samples and both in the *Sham Operated group* and base level.

Change in white blood cell: In the portal venous blood sample of the Ishaemia-Reperfusion group, the *white blood cell* showed elevation increase (3.45±0.56 x 10^3/μL), which was significant in the 30th (4.81±2.02 x 10^3/μL) and 60th (5.0±0.77 x 10^3/μL) minutes of reperfusion compared to the result of Sham Operated group (2.18 ± 0.42 x 10^3/μL, $p<0.001$ and 2.18 ± 0.14 x 10^3/μL, $p<0.001$)

In the caudal *caval vein* blood samples of *Ischemia-Reperfusion group* we obtained similar results: white blood cell showed elevation in the 15th min of reperfusion (4.58 ±1.49 x 10^3/μL) compared with the base value (3.7 ± 0.52 x 10^3/μL). The elevation was significant in the 30th and 60th min of the reperfusion (4.36 ± 1.03 and 5.86 ± 0.70 x 10^3/μL, $P < 0.001$, respectively) compared with the base level and to the *Sham Operated group* (2.58 ± 0.36 and 2.05 ± 0.82 x 10^3/μL; $p < 0.05$)

In *Sham Operated group*, a continuous decreasing was observed, reaching significantly ($p<0.05$) lower values at 30^{th} and 60^{th} minutes versus control base level.

5.3. DISCUSSION

Results showed the worsening of red blood cell deformability and aggregation, as well as during reperfusion hematocrit, mean corpuscular volume and red blood cell was significantly elevated in the *local circulation* (portal vein) and *systemic circulation* (caval vein).

We confirmed the earlier findings that the observed changes in hemorheological parameters significantly affect the microcirculation after ischemia, which can additively increase the damage in areas previously excluded from the circulation.

Red blood cell deformability significantly altered by the 30^{th} and 60^{th} min of reperfusion both in the systemic (caudal caval vein) and local (portal vein) blood, since $SS_{1/2}$ increased in these given times.

In a previous canine study (4^{th} chapter), using bulk filtrometry method, we found alike impairing of red blood cell deformability in the systemic blood at 2^{nd} hour of the reperfusion after 30 min ischemia (induced by clamping the superior mesenterial artery), in which red blood cell deformability worsened during mesenterial ischemic reperfusion. *The results obtained with the newer ektaytometric method confirmed the importance of red blood cell deformability in case of ischemic-reperfusion damage*

The explanation of the worsening of red blood cell deformability could be the local changes in the factors of microenvironment, such as the ion level, the pH, the osmolarity, the decrease of intracellular ATP level and altered intra- and extracellular fluid distribution Change in these factors could affect the red blood cell shape and volume. Red blood cell deformability can be further decreased due increase production of reactive oxygen species by lipid peroxidation, modified enzymes, changed protein functions or pathologically altered hemoglobin molecules.

The red blood cells passive deformability plays an important role in the pathway of blood cells through the microcapillary. In the samples of *Ischemia-Reperfusion group* from both circulations, the measured significantly increase in MCV could contribute to the worsening of red blood cell deformability through the caused change in the shape and volume of the red blood cells. The significant increase of hematocrit also could contribute to this pathway in the *Ischemia-Reperfusion group* samples of local and systemic circulation.

By analyzing our results, we found increase in *red blood cell aggregation* compared to base level at the end of the 30 minutes ischemia in the portal vein sample. This showed further increase during the reperfusion compared to the caval blood sample. In the local (portal vein) blood samples, we observed approximately 2.5-fold increase of aggregation at the end of reperfusion compared to the systemic (caval vein) samples with same times.

Increase of aggregation capability of red blood cells is well know in acute MI, in inflammation, or in case of trauma. However, we have not found available information on the changes of these parameters during ischemia-reperfusion.

It is known from the experiments of Baskurt et al., if red blood cells are placed into a superoxide anion, produced by xanthine oxidase hypoxanthine, containing medium, then it primarily affects the aggregation capability of red blood cells rather than the deformability. The same experimental group found the increase of red blood cell aggregation during ischemic reperfusion due to the decrease in cell surface charge. If the surface charge of the cells change, then the electrostatic repulsion force also decrease, which may deviate the blood cells to aggregate.

In our study, white blood cell count was found to be elevated in the portal and caval vein and this at the 30th and 60th minutes of reperfusion. Especially the increase of polymorphonuclear cells is the major importance, but lymphocytes also have an important role in development of tissue/ischemic-reperfusion injury. In addition to increased rigidity, PMN activation is associated with an increased level of secretor activity, resulting in the production and release of chemokines and cytokines, reactive oxygen species, proteolytic enzymes by the cell and lead to tissue destruction. The effectively of the process gradually decrease from the epicenter.

The activated polymorphonuclear leukocytes can increase the free radicals produced during the enzymatic reaction of xantin-oxidase system, which can be found high concentration in the intestinal mucosa. It enhances the harmful effects of these processes.

In summary, we can conclude that during mesenterial ischemia-reperfusion the hematocrit, red blood cell deformability, red blood cell aggregation, *white blood cell*, mean corpuscular volume values show change in the first hour of reperfusion in the local and systemic samples. The hemorheological investigations can be useful for further understanding the complex pathomechanism of changed hemorheological parameters during ischemic-reperfusion, and can provide information, which can be helpful in clinical treatment and faster rehabilitation of patients, associated with mesenterial ischemia.

6. "AVASCULAR" SMALL BOWEL TRANSPLANTATION IN MICE - IV. EXPERIMENTAL MODEL

6.1. MATERIAL AND METHOD

6.1.1.Experimental animals and anesthesia

The experiment was approved by the University of Debrecen Committee of Animal Research (permission number: 6/2001 UDCAR). Thirty-six A/J mice weighting 20-24 g (22.32 ± 1.66g) were used in the experiment with microsurgical procedure.

Atropine (1% atropium sulfuricum, 0.05 mg/kg, PAINES&BYRNE Ltd.) was used for premedication subcutaneously. Anesthesia was performed using Nembutal (sodium-pentobarbital 35 mg/kg, PHYLAXIA-SANOFI Ltd.) intraperitoneally.

6.1.2. Operative Technique, experimental groups

6.1.2.1. Donor Operations

The interventions were performed on A/J inbreed mice. Donor operation (n=6): after depilation of animals, disinfection with Betadine and isolation, a median laparatomy was performed. Incision was made with a scissor next to the xyphoideus process (which is a plate shaped structure in mice) on the right and left side until reaching the ribs. Distally, we opened the abdominal cavity until the area of pubic symphysis with a special attention not to injure the bladder. Sterile gauze sheets were used for the isolation of the surgical area.

The small bowels were lifted with a wet swab and were placed on a gauze sheet saturated the body warm physiological saline solutions. We chose a 2 cm long jejunum segment from 2 cm of the Trietz ligament, and it was excised with a scissor. The jejunum segment was cannulated with a polyethylene catheter, and then it was slowly washed. For the washing, we diluted a proampulla of Neomycin-sulfate (Neomycin Sulfate, (600 IU/mg), Merck, Ltd.) with 4ºC Ringer lactate solution to 20 ml. The advantage of the chosen antibiotic is that it is not absorbable in the small bowel. We washed the graft until the washing solution became clean. The bowel segment was open alongside and several small 3x2x2 mm pieces were sliced with a scalpel. The small bowel slices were kept in lactate Ringer's solution at 4ºC until their utilization.

6.1.2.2. Recipient Operation

During the recipient operation (n=30) and after the median laparatomy the large omentum was raised and placed on wet gauze. Five nests were prepared by opening the front layer of the omentum. The nests were made near the omental vessels, but with a special care to avoid injuries of the vessels. Small bowel pieces were taken into all nests, between the two layers of omentum. They were closed with 8/0 Prolene (prolyprpylene) stitches, which were also marking the location of the nest. The abdominal cavity was closed in two layers. To decrease the damage caused by ischemia-reperfusion, the bowel segement were stored for a shorter period and each donor operation was followed by 5 recipient operations.

Six weeks after the surgical procedures, biopsy was done for histological investigations. The operation and the histological sampling were done at the same time of the day, and with similar circumstances (between 9:00- 11:00).

6.1.3. Macroscopic investigation

At the end of the investigated period but before the biopsy taking, the abdominal cavity of the experimental animals was carefully explored, the operated area was examined and video and photo documentation were taken.

6.1.4. Microscopic investigation

Biopsy materials were stained with hematoxylin and eosin (H&E), periodic-acid-Schiff (PAS), and Van Gieson.

6.2. RESULTS

All animals survived the experimental period, until the time of tissue sampling. The average donor operation time was 15 ± 2 minutes and the recipient operation took 25 ± 3 minutes.

6.2.1.Macroscopic investigation

During the macroscopic analysis we could not find 7 marked nests. 9 cases we observed adhesion to the abdominal wall and in 4 cases we found adhesion to the liver. In the other cases we found all the nest with transplanted small bowel segments in the greater omentum. In several cases, as seen in the figure we found the transplanted bowel segments with water-clear, transparent, cystic lesions. In most of the cases cystic lesion was not formed, but the transplanted bowel chips embedded like an island in the greater omentum.

6.2.2. Microscopic investigation

We identified the bowel chips transplanted into the greater omentum during histological investigation. All bowel chips were highly vascularized. The intestinal wall flattened, but its entire layer: musosa, submucosa, muscular layers, subserosa, and serosa were visible. In the transplanted tissue the glands seemed to be functioning. Lymphocytic infiltration was seen in the muscular layers.

With the help of PAS stainin, the mucin produced by the mucus producing glands became visible and also these mucin producing goblet cells could be identified.

The water-clear, transparent, cystic lesions could be identified with the dilated, flattened layers of the intestinal wall during the histological investigation. The mucosa located in one layer and in some places detached from the wall. Goblet cells were detected in the mucosa. The muscular layer flattened. The muscle layer was slightly infiltrated by lymphocytes. The transplant

was surrounded by a moderate mononuclear cell infiltrate with some giant cells. The lumen was filled with mucinous fluid.

6.3. DISCUSSION

All the layers of the intestinal wall could be identified on the slides. The preserved mucosal glands retained its *mucin producing* ability and several *goblet cells* could be detected. Transplantation was successful in 23 recipients.

During our experiment, we tried to find the most suitable place for the implantation of the bowel chip and to reduce the postoperative complications.

Based on previous publications, researcher tried to transplant bowel segments into various places of the body. Uchida et al. transplanted 1.5 cm small intestinal segment with a completely removed mesentery *into the subcutan layer of the abdominal wall* and all the layers could be identified two weeks after the operation. They established 75% of revascularization.

Lane et al. transplanted a 30 mm small intestinal segment into the space created between the *parietal peritoneum and the rectus abdominis muscle*. After 10 days the graft survival rate was 90% at the biopsy, however several cases the structure of the bowel wall was damaged.

Tisinai et al. used 2 cm jejunum and ileum segments. In one group they implanted the segment *under the capsule of the kidney*, in the other group they were wrapped around with the *greater omentum*. After two weeks, they found the transplanted intestinal grafts larger, but the difference was not significant.

The most suitable place for transplantation is the greater omentum. It was demonstrated in our previous spleen autotransplantation experiment in mice, which provided the basic idea for this small bowel transplantation model.

First Klos et al. used the greater omentum for bowel transplantation, but they just covered the intestinal segment and the homograft with the greater omentum. After 6 weeks of the operations the muscular layer was identified all the time, but he bowel wall layers were found in fewer cases. They experienced rejection of the homografts.

During our operative technique the anterior layer of the greater omentum was incised and the intestinal segment were placed into these nests then they were fixed with stitches, not just covered with the greater omentum.

Three characteristics make the greater omentum useful for transplantations: portal vein circulation, antibacterial effect of the greater omentum (“protector of the abdominal cavity), and mainly its special revascularizing ability. Levy et al. proved the neoangiogenic effect of angiogen factors extracted from the grater omentum.

Experiments conducted on mice have several general advantages compared to other experiment animal species:

1./ *Numerous genetically modified mouse strains* are known. Congenic transgenic and knock out strains exist, which help to create experimental models for investigation of acute, chronic rejection, and graft versus host diseases.

2./ Several *monoclonal, polyclonal* antibodies are known for inbreed and outbreed mouse strains.

3./ Because of smaller body weight *smaller amount* of the medicine, chemicals, reagents and antibodies are used.

4./ The *maintenance costs* are lower compared to other animals. Mice have smaller space requirements and greater reproductive ability.

The advantage of *our designed experimental model*:

1./ Execution of a simple, relatively fast method of operative technique
2./ There is not need for vascular anastomosis
3./ More then 10 transplantation can be done effortlessly in a day, compared with the classical organ transplantation, which requires vascular anastomosis.
4./ This model may be suitable for testing newer immunosuppressant medication, and research in stem cells.
5./ It is ideal for research of xenotranplantation, when different species' different sized organs transplantation will take place.
6./ *Difference in size of the organs is not a problem*, important advantage.
7./ It can be used for transplantation of different organs, not just for only one organ.

7. DEVELOPING FURTHER THE CURRICULUM OF BASIC MICROSURGICAL TRAINING TO TRANSPLANTATION RESEARCH.

There are increasing numbers of diverse researches, which require procedures done under microscope on smaller laboratory animals (rat and mouse). Therefore, there is a greater necessity for well-trained and experienced professionals in microsurgical operative technique, since the application of microsurgical methods minimizes the risks of the interventions and increases the safety of surgery.

7.1. Educational curriculum required for acquisition of basic microsurgical technique

To decrease the difficulty of the advanced microsurgical operative technique of small bowel transplantation in mice and the number of complication caused by the transplantation we developed a simple microsurgical model, which acquisition is enough with basic training in microsurgery.

Furka microsurgical educational method is the acquisition of basic microsurgical technique, which features activity, synchronism, video-assistance, self-controlling, individualization, analysis.

The *acquisition of basic technique* is during the so-called "dry training", where *the thematic of the practices* are the following: *in the first practice* we review the history of microsurgery, application field, and the role of small laboratory animals in microsurgery. During *the second practice* we introduce the operative microscope, the microsurgical instruments –scissors, needle holders, forceps, approximator-, the microsurgical suture materials and the needles.

Afterwards *the operating room practices* follow, during which the first lesson is to establish the harmony between the eyes and hands under the microscope with different magnifications. The exercise of next practice helps the safety acquisition of the depth sensations: scratching letter from a newspaper, guiding gauze fiber with microsurgical instruments. It is followed by the acquisition of microsurgical suturing and knotting technique: stitching into a rubber glove, closing the incisions made on the glove, making suture lines in different directions. To make a vascular suture line and an end-to-end vascular anastomosis on a biomodel prepared from the femoral artery of experimental animals.

In the following, *the practice is performed on living tissues*. The task is to reconstruct the abdominal aorta of the rat: performing median laparatomy after intraperitoneal anesthesia, preparing the infrarenal section of the abdominal aorta, and then creating an end-to-end vascular anastomosis according to the previously practiced technique on the "dry-training".

Usually, the participants reach this level up to now during the basic course. *The above-mentioned method gives enough microsurgical skills for the researchers, so they can safely perform our described model.*

In case the participants have been acquainted with the basic microsurgical technique, then they can move forward step-by-step to more serious microsurgical operations, which requires advanced technical preparedness.

Next to the abdominal aorta of the rat, the reconstruction of the caudal vena cava represents a new difficulty level. This can be followed by the practicing and acquisition of end-to-end, end-to-side vascular anastomosises on the femoral artery, then on common carotid artery.

The so-called "dry-training" promotes the practicing on the living tissue, this is also an animal welfare method, and it ensures next to respect of life the basic principle of the Law of Animal Protection –*the 3R rule*-: Reduction, Refinement, and Replacement.

7.2. Educational curriculum for acquisition of small intestinal transplantation requiring advanced microsurgical technique.

For a better understanding and comparison, I present the originally described method of the microsurgical "vascular" small intestine transplantation model –using vascular anastomosises- by Zhong with our developed "avascular" small intestinal transplantation model in mice.

The essence of the method, main steps of the operative technique:

In the *donor operation*, after median laparatomy, as a first step the large intestine and ileum was resected, and then the jejunum was identified and the mesenterial vessels were ligated. The portal vein was carefully dissected from the pancreas, then the aorta was prepared and he celiac trunk was ligated. Furthermore, the renal vein was also ligated. The infrarenal segment of the abdominal aorta was punctured with a fine needle, and the selected graft was washed out with 0.2-0.4 ml 4 °C Ringer-lactate with Heparin solution. Afterwards, the portal vein was cut near the hilum of the liver. The aorta was also incised according to the Carrel patch technique, and then the graft was wrapped in wet gauze and placed into icy, 4 °C Ringer-lactate, and stored until further usage.

In the *recipient operation*, after median laparatomy, the infrarenal section of aorta and caudal vena cava was carefully prepared, then 1 cm long segment of the aorta and vena cava was debarred from the circulation with the help of microsurgical clips. An elliptic shaped arteriotomy and venotomy was performed, and the excluded vessel segments were washed with physiologic saline solution with heparin. The donor small intestinal segment was placed near the appropriate position in the recipient.

First of all, the veins of the donor and recipient were united with *end-to-side anastomosis* with the help of 11/0 Prolene (polypropylen) suture materials, paid careful attention that the length of the opening of the elliptic shaped venotomy of the recipient exceeded the diameter of the caudal vena cava of the donor. Inverting stitches were used on the posterior wall unlike the applied statutes in vascular surgery –during a vascular anastomosis always the intima layers are faced to each other- sutured from the lumen of the vessel, and then the suture of anterior wall was made with the proper everting stitches. The *end-to-side anastomosis of the arterial branch was* created by using 11/0 Prolene continuous suture line.

One of the possibilities of finishing the operation was that the transplanted small intestine was lead out to the abdominal wall with two stomas, leaving the original intestinal segment intact. The other possibility was that between the transplanted intestinal segment and the original

jejunum an end-to-side anastomosis was made, while the other end of the graft was created into a stoma. The abdominal cavity was closed with one layer.

The operation requires large experience in the field of microsurgery. Before the learning of the technique of small intestine transplantation, the execution of end-to-side anastomosis (within 10-15 minutes) must be acquainted in a routine manner. In the learning phase, 100 transplantations are suggested to make on mice with average weight of 20-23g, so the complication of the operative technique should not effect the investigations followed the transplantation.

What complication could be? *The possible complication of small intestinal transplantation:*

Hypovolemic and septic shock: shock is the most general complication of small intestinal transplantation, which might lead to death of the recipient within the fist 48 hours. The manipulation during the operation can easily injure the structure of the bowel wall, and also during the washing of the intestinal tract with using of high pressure can easily cause damage. The emerging harmful effects during the ischemia-reperfusion ca block the physiologic function of the intestinal wall, contributing to fluid loss, translocation of bacteria, which might lead to hypovolemic and septic shock.

Arterial thrombosis: it is the most general late complication of small intestinal transplantation. Experiences have shown that the appropriate surgical technique is more important in the avoidance of the complication then the treatment with anticoagulant. It is suggested to make the anastomosis with non-absorbable suture material, continuous suture line and in short time, in contrast to simple stitches, which take longer time.

Stoma necrosis: this complication appears after the operation 4-7 days, generally a mechanic injury, due to inappropriate technique.

To avoid these complications, many-many experiences in microsurgical proficiency are required, ensuring the implementation of a successful animal model.

The listed complications propounded the idea to try our previously developed spleen autotransplantation model, with small modifications, to create an “avascular” type small intestinal transplantation, which was previously described in section 6.1.2.

8. SUMMARY OF THE MAIN RESULTS AND CONCLUSIONS

1. We implemented a *new operative technique- a double, isolated jejunum-segment model-* to investigate the damages caused by mesenterial-ischemia reperfusion on mixed breed dogs. One of the segments was used for carrying out the investigation of ischemia-reperfusion after atraumatic vessel compression, while the other one was the control without compression.

2. We were the first to *describe the apoptotic process due to ischemia-reperfusion in the mucosa of the small intestine during the fist 24 hours after the operation* on mixed breed dogs. Apoptotic cells were detected from the 30th minute of reperfusion in the double, *isolated jejunum-segment* model after 30 minutes of ischemia; the *most extent was in the 4th and 6th hours of reperfusion.*

3. On the double, isolated jejunum-segment model, we successfully investigated the effectiveness of different *ischemic preconditioning* protocols. We concluded, that the 3x5 minute ischemic preconditioning protocol significantly increased the *apoptotic activity.*

4. We were first to describe, that during the pretreatment of *Vitamin E (*25mg/kg) *and allopurinol* (50mg/kg) the *apoptosis increased* in the intestinal mucosa during *ischemia-reperfusion.*

5. In conclusion, if *the ischemic insult was milder* or certain protective *factors (preconditioning, allopurinol, Vitamin-E)* were present, then the *ischemic cell death mainly caused by apoptosis*. Inflammation and tissue damages presented to a lesser extent in the tissue, *by this providing a favorable condition for tissue regeneration.* The results found in our model could explain the hypothesis of the related clinical articles, in which the blocking the activation of NF-κB by Vitamin E could enhance apoptosis in the mucosa of the intestine during the damages caused by ischemia-reperfusion.

6. In relation to the results of hemorheologic filtrometric investigation during 30 minutes of compression of the superior mesenteric artery in mixed breed dogs, we were the first to draw attention to the importance of the *investigation of red blood cell deformability* on the *3rd postoperative day*, which could provide indicative information on the state of intestinal surgery, the extent of the damage of ischemia-reperfusion associated with the intervention, early sign of the intestinal involvement and *the possible damage of microcirculation.*

7. We were the first to draw attention to the *rheologically significant worsened blood quality flowed from the mesenterial region to the rest of the body during* the hemorheologic ektacytometric and aggregometric investigations of 30 minutes of mesenterial ischemia and the following 60 minutes of reperfusion on outbred rats. We proved the local significant deterioration of several hemorheologic parameters *-red blood cell deformability, red blood cell aggregation, hematocrit, mean corpuscular volume-* compared to systemic circulation. These hemorheological investigations may be beneficial in the understanding of the complex pathomechanism of changed rheologic parameters due to ischemia-reperfusion and may provide informations, which might be helpful for the treatment of patients in a critical state due to mesenterial ischemia and their faster rehabilitation.

8. *New, cost-beneficial, "avascular" small intestinal transplantation model* was created in *inbred mice* with the partial modification of our previously developed spleen autotransplantation technique.

9. The viability of small intestinal segments in the *greater omentum* was proven by macroscopic and microscopic investigation with the application of the *"avascular" small intestinal transplantation technique*. All the layer of the intestinal wall was detected. We proved the functional activity of the glandular epithelium of the transplanted bowel segments with the detection of mucin production. The model may be suitable for the testing of newer immunosuppressant medicine, stem cell and xenotranplantational research.

10. We developed a microsurgical teaching curriculum for small intestinal transplantation model on mice, which require advance microsurgical technique, at the same time drawing attention to the possible complications.

9.1. Ph.D. thesis based in extenso articles

1. **Bráth E.**, Kovács J., Mikó I., Komár T., Furka I.: Apoptosis caused by ischaemia-reperfusion in the intestinal wall of laboratory animals.
   Acta. Chir. Austr. 1998;30(Suppl. Nr. 147):45-46.

2. **Bráth E.**, Furka I., Németh N., Szabó Gy., Pető K., Ács G., Lesznyák T., Cserni T., Pap Szekeres J., Mikó I.: Changes of the red blood cells deformability caused by mesenteric ischemia-reperfusion injury. An experimental animal study.
   In: Proceedings of the 37th Congress of the European Society for Surgical Research, 2002. pp. 281-285. Ed: Boros M., Monduzzi Editore, Bologna

3. Mikó I., **Bráth E.**, Furka I., Kovács J., Kelvin D., Zong R.: Spleen autotransplantation in mice: A novel experimental model for immunology study.
   Microsurgery 2001;21:140-142. **IF: 0,844**

4. Mikó I., **Bráth E.**, Furka I.: Basic teaching in microsurgery.
   Microsurgery 2001;21:121-123. **IF: 0,844**

5. **Bráth E.**, Mikó I., Kovács J., Tóth F. F., Fachet J., Furka I.: Multiorgan transplantation with a new organ-chip technique in mice. Preliminary histological data.
   Microsurgery 2003;23:466-469. **IF: 0,711**

6. **Bráth E.**, Németh N., Papp L., Békési L., Varga S., Tóth A., Mikó I., Furka I.: Mikrosebészeti oktatás a kutatás és a klinikai gyakorlat szolgálatában.
   Magy. Seb. 2005;58:111-115.

7. Furka I., **Bráth E.**, Németh N., Mikó I.: Learning microsurgical suturing and knotting techniques: comparative data.
   Microsurgery 2006;26:4-7. **IF: 0,882**

8. **Bráth E**., Németh N., Kiss F., Sajtos E., Hevér T., Mátyás L., Tóth L., Mikó I., Furka I.: Changes of local and systemic hemorheological properties in intestinal ischemia-reperfusion injury in the rat model.
   Microsurgery 2010;30:321-326. **IF: 1,244**

9. **Bráth E**., Németh N., Kovács J., Pető K., Mikó I., Furka I.: Effects of allopurinol and preconditioning on apoptosis due to ischemia-reperfusion on a double jejunum-segment canine model.
   Acta. Vet. Hung. Közlésre elfogadva **IF: 0,479**

The cumulative impact factor of the Ph.D. thesis based in extenso articles: 5,004

9.2. In extenso articles related to this Ph.D. thesis

1. Schwabegger A. H., Kronberger P., Obrist P., **Bráth E.**, Mikó I.: Functional Sphincter Ani Externus Substitute Using Free Innervated Latissimus Dorsi Muscle (LDM) Flap: an Experimental Study in Dogs.
   In: Reconstructive Microsurgery 2001. pp. 43-44.
   Eds.: Fu-Chan Wei, David C. C. Chuang, Hung-Chi Chen, Monduzzi Editore, Bologna

2. Pap Szekeres J., Cserni G., Furka I., Svebis M., Cserni T., **Bráth E.**, Németh N., Mikó I.: Transplantation and microsurgical anastomosis of free omental graft. Experimental animal model of a new operative technique in dogs.
Microsurgery 2003;23:414-418. **IF: 0,711**

3. Németh N., Lesznyák T., **Bráth E.**, Ács G., Nagy Á., Pap Szekeres J., Furka I., Mikó I.: Changes in microcirculation after ischemic process in rat skeletal muscle.
Microsurgery 2003;23:419-423. **IF: 0,711**

4. Németh N., Szokoly M., Ács G., **Bráth E.**, Lesznyák T., Furka I., Mikó I.: Systemic and regional hemorheological consequences of warm and cold hind limb ischemia-reperfusion in a canine model.
Clin. Hemorheol. Microcirc. 2004;30:133-145. **IF: 0,630**

5. Sipka S. Jr., **Bráth E.**, Tóth F.F., Fábián Á., Furka I., Mikó I.: Lép autotransplantatio és lépeltávolítás haematologiai és immunologiai hatásainak összehasonlító vizsgálata egerekben.
Magy. Seb. 2005,58:84-88.

6. Pap Szekeres J., Pető K., Németh N., Cserni G., Furka I., Svébis M., Cserni T., **Bráth E.**, Mikó I.: Extraabdominalisan átültetett cseplesz lebeny mikrocirkulációjának intraoperatív vizsgálata laser Doppler flowmetria segítségével kutyán.
Magy. Seb. 2005;58:116-119.

7. Pető K., Oláh V. A., **Bráth E.**, Németh N., Gulyás A., Szilasi M., Sárvári M., Furka I., Mikó I.: Ischaemia- reperfusiós vesekárosodás és az Allopurinol védő hatásának kimutatása vizelet NAG követésével.
Magy. Seb. 2005;58:134-137.

8. Mikó I., Németh N., Sipka S. Jr. **Bráth E.**, Pető K., Gulyás A., Furka I., Zhong R.: Hemorheological follow-up after splenectomy and spleen autotransplantation in mice.
Microsurgery 2006;26:38-42. **IF: 0,882**

9. Sipka S. jr., **Bráth E.**, F Toth F.,Aleksza M., Kulcsar A., Fabian Á., Barath S., Balogh P., Sipka S., Furka I., Miko I.: Cellular and serological changes in the peripheral blood of splenectomized and spleen autotransplanted mice.
Transpl. Immunol. 2006;16:99-104.
IF: 2,297

10. Furka I., **Bráth E.**, Németh N., Mikó I.: Gondolatok a mikrosebészeti oktatásról. Mire volt elég az 5460 óra a mikrosebészeti alapoktatásban?
Magy. Seb. 2006;59:147-151.

11. SchwabeggerA. H., Kronberger P., Obrist P., **Bráth E.**, Mikó I.: Functional sphincter ani externus reconstruction for treatment of fecal stress incontinence using free latissimus dorsi muscle transfer with coaptation to the pudendal nerve: preliminary experimental study in dogs.
J. Reconstr. Microsurg. 2007;23:79-85. **IF: 0,722**

12. Pető K., Németh N., **Bráth E.**, Takács E. I., Baskurt O. K., Meiselman H. J., Furka I., Mikó I.: The effect of renal ischaemia-reperfusion on hemorheological factors: preventive role of allopurinol.
Clin. Hemorheol. Microcirc. 2007;37:347-358. **IF: 0,977**

The cumulative impact factor of the in extenso articles related to this Ph.D. thesis: 6,93

9.3. Other in extenso articles

1. Incze D., **Bráth E.**, Pető K., Kovács J., Kuhnyár Á., Sefcsik I., Furka I.: Laparolift használata utáni hasfalelváltozások kísérletes vizsgálata.
   In: Bibliomed Medizinische Verlagsgesellschaft mbH. 2000. pp. 11-16.

2. Szűcs G., Tóth I., **Bráth E**., Gyáni K., Mikó I.: Nyelőcsővel készült süllyesztett és más gyakrabban alkalmazott anasztomózis változatok belső nyomástűrésének vizsgálata modell kísérletekben.
   Magy. Seb. 2001;54:239-244.

3. Szűcs G., Tóth I., **Bráth E**., Gyáni K., Mikó I.: Adatok a nyelőcső és a gyomor reszekciós műtéteinél kézzel varrt anasztomózisok korai fizikai varratbiztonságához.
   Magy. Seb. 2001;54: 325-330.

4. Szűcs G., Barna T., Tóth I., **Bráth E.**, Gyáni K., Incze D., Mikó I.: Süllyesztett anasztomózisok gyógyulásának vizsgálata állatkísérletekben.
   Magy. Seb. 2003;56:73-79.

5. Szűcs G., Tóth I., Barna T., **Bráth E.**, Gyáni K., Mikó I.: Operation technique and healing process of telescopic ileocolstomy in dogs.
   Acta Vet. Hung. 2003;51:539-550. **IF: 0,535**

6. Szűcs G., Tóth I., Barna T., **Bráth E.**, Gyáni K., Mikó I.: Experimental examination of the healing process of the telescopic esophageal anastomosis.
   Dis. Esoph. 2003;16:229-235. **IF: 0,809**

The cumulative impact factor of other in extenso articles: 1,344

The cumulative impact factor of all articles in extenso: 13,278

The cumulative impact factor of all in extenso articles with the articles not presented in this Ph. D. thesis: 33,248