FINAL REPORT - Grant Nr. K-105618

COMPLEX FOLLOW-UP INVESTIGATIONS OF FILTRATION AND IMMUNOLOGICAL FUNCTION OF THE SPLEEN AFTER DIFFERENT TYPES OF SPLEEN-SPARING SURGICAL TECHNIQUES (MORPHOLOGICAL EXAMINATIONS WITH FUNCTIONAL IMAGING DIAGNOSTICS AND STEM CELLS, MICRO-RHEOLOGIC AND IMMUNOLOGIC METHODS)

Irén Mikó[1], István Furka[1], Norbert Németh[1], Katalin Pető[1], Ferenc Kiss[1], Enikő Tóth[1], Gergely Kalóczkai[1], Ádám Deák[1], Erzsébet Ványolos[1], Viktória Sógor[1], Mátyás Kiss[2], Bence Dorogi[2], András Szuák[2], Ágnes Nemeskéri[2], Andrea Furka[3], György Trencsényi[4], Ildikó Garai[4], József Varga[5], László Tóth[6], Krisztián Szigeti[7], Ilona Benkő[8], Anna V. Olah[9]

[1]Department of Operative Techniques and Surgical Research, Faculty of Medicine, University of Debrecen,
[2]Department of Anatomy, Histology and Embriology, Clinical Anatomical Research Laboratory, Faculty of Medicine, Semmelweis University, Budapest,
[3]Department of Clinical Oncology, Division of Radiotherapy, Faculty of Medicine, University of Debrecen,
[4]Department of Medical Imaging, Faculty of Medicine, University of Debrecen, Debrecen,
[5]Department of Nuclear Medicine, Faculty of Medicine, University of Debrecen, Debrecen,
[6]Department of Pathology, Faculty of Medicine, University of Debrecen, Debrecen,
[7]Department of Biophysics and Radiation Biology, Faculty of Medicine, Semmelweis University, Budapest,
[8]Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen,
[9]Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary

1. INTRODUCTION

The spleen is a very important immune organ, which prevents spread of pathogens from gastrointestinal system. It has complex immune functions which have to be preserved as much as it is possible. If we have to remove spleen we have to preserve healthy parts of spleen as much as it is possible.

Filtration, immunological and storage function of the spleen has been revealed in the past decades. Loss of these functions by the removal of injured but functionally intact spleen after splenic trauma may have serious consequences and lead to the often fatal complications.

The protection against infections can decrease. After the splenectomy approximately in 3% of adults and in 6-7% of children Overwhelming Postsplenectomy Infection (OPSI) and/or disseminated intravascular coagulopathy (DIC) syndrome can occur with 65-70% mortality rate.

To save splenic function, spleen-autotransplantation technique into the greater omentum was developed by Furka. Partial or subtotal spleen resection or spleen autotransplantation can also partly preserve and restore the splenic filtration and immunological function, as previous studies by Furka's method also demonstrated.

In recent grant we aimed to follow-up the long-term effects of splenectomy, spleen-autotransplantation (spleen „chips" placed in between the sheets of the greater omentum) and spleen resections at various degree on beagle dogs using complex investigative methods.

These comparative investigations maybe could indicate the diagnostic markers of possible OPSI or/and DIC syndromes based on asplenic or hyposplenic state after traumatic spleen injuries with splenectomy or splenic sparing surgery.

2. MATERIALS AND METHODS

2.1. Experimental animals, narcosis and operative techniques

Experimental animals, permission:

The experiments were approved by the University of Debrecen Committee of Animal Welfare (permission Nr.: 26/2011/UDCAW) in accordance with the national regulations (Law XXVIII/1998) and EU directives (2010/63).

Twenty-six healthy male and female beagle dogs (age: 18-23 months old, 19.4±1 months, bodyweight: 12.98±1.1 kg) were involved in this study.

Narcosis:

All of the operations were performed in sterile condition under narcosis with intramuscular ketamine (10mg/bwkg, CP-Ketamin – ketamine hydrochloride 10%, Produlab Pharma B.V., Netherlands) and xylazine (1 mg/bwkg, CP-Xylazin – xylazine-hydrochloride, 2%, Produlab Pharma B.V., Netherlands) combination.

Experimental groups and operative techniques:

I-II. *Control groups* (C, n=6): This group contains the *sham-operated* (n=3) *and healthy control* (n=3) animals as well. In sham-operated control animals (n=3) midline laparotomy was performed, while in healthy controls (n=3) without any surgical intervention.

III. *Splenectomy group* (SE, n=4): after midline laparotomy the spleen was removed.

IV. *Spleen-autotransplantation group* (AU, n=8): after median laparotomy and total splenectomy, ten pieces of splenic slices (20 mm x 50 mm x 1 mm) were placed between two layers of the greater omentum, close to a well-vascularized area according to the"Furka's spleen chip/apron" method.

V-VI. *One-third* (partial resection) *and two-third* (subtotal) *spleen resection groups* (R1/3 and R2/3, n=4/each): After median laparotomy one-third or two-third part of the distal region of the spleen was resected) using „Furka-type" embracing suture technique.

In every operated group the abdominal wall was sutured in two layers. First the muscle and peritoneum were closed with 1-0 polyglycolic acid absorbable suture material (Optime®, Peters Surgical, France) using simple interrupted stitches, then to close the skin 2-0 polyglycolic acid absorbable suture material (Optime®, Peters Surgical, France) using Donati vertical mattress stitches were used.

For certain investigative examination we also calculated with data from previous K-49331 OTKA grants' results. These included 26 months beagle model follow up results as well. (C, n=6, SE, n=4, AU, n=8, R1/3, n=4, R2/3, n=4).

The results of these two investigation periods involved 52 experimental animals in total.

2.2. Blood sampling protocol

Regarding the presented parameters in this experimental work, blood samples were taken before the operations (base) and in the 1^{st}, 3^{rd}, 6^{th}, 9^{th}, 15^{th} and 18^{th} postoperative months via puncturing the cephalic vein using closed blood sampling system with 21 G BD EclipseTM needles (Becton, Dickinson and Company, USA). For hematological and micro-rheological investigations K3-EDTA was used as anticoagulant (1.8 mg/ml, BD Vacutainer®).

2.3. Testing hematological parameters

The Advia 120 (Siemens Healthcare GmbH, Germany) and/or Sysmex F-800 hematological automatic devices system was used to determine quantitative and qualitative hematological parameters. In this grant white blood cell count (WBC [G/l]), red blood cell count (RBC [T/l]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin concentration (MCHC [g/dl]), hematocrit (Hct [%]), hemoglobin (Hgb [g/dl]), mean corpuscular hemoglobin (MCH [pg/l]), CHCM [g/dl], CH ([pg]), red cell distribution width (RDW [%]), hemoglobin distribution width (HDW [g/l]), platelet count (Plt [G/l]), mean platelet volume (MPV [fl]) were analyzed. Changes in these parameters may affect the assessment of changes in the red blood cell deformability. These measurements required blood sample volume of 175 µl.

We also investigated the effects of splenectomy and autotransplantation on serum lipid profile (HDL-C, LDL-C, total cholesterol, triglyceride) and the changing of different enzym's levels (LDH, GOT, GPT, GGT, AP) and IgM level, too.

2.4. Measuring fibrinogen concentration

Fibrinogen concentration (Fbg [g/l]) of the plasma was determined in plasma from blood anticoagulated with sodium-citrate (0.129 M, BD Vacutainer® tubes) using a Sysmex CA-500 coagulometer (TOA Medical Electronics Co., Ltd., Japan) based on the Clauss's method.

2.5. Micro-rheological investigations

2.5.1. Determining red blood cell aggregation with two different methods

2.5.1.1. Light-transmittance aggregometry

For testing the red blood cell aggregation a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) was used. The technique is based on light-transmittance photometric method. The test requires approximately 20 µl of blood. During the measurements the sample is disaggregated (at 600 s-1) then the shear rate drops to zero (M mode) or to 3 s-1 (M1 mode). According to the changes in light-transmittance the instrument calculates the aggregation index values at the 5th or 10th second of the process. The indices (M 5s, M 1 5s, M10 s, M1 10s) increase with enhanced red blood cell aggregation.

2.5.1.2. Syllectometry

In parallel with the light-transmission technique, erythrocyte aggregation was also tested with a LoRRca MaxSis Osmoscan ektacytometer (Mechatronics BV, Hollandia) operating with laser-backscattering method. The tests require 1 ml of blood.

After disaggregating the blood sample with rotation in the Couette-system, the rotor stops and the changes in the intensity of the light reflected from the sample is measured. From the intensity-time curve the software calculates several parameters, including aggregation index (AI [%]), amplitude (Amp [au]) and aggregation half time (half-amplitude time, t1/2 [s]), describing the rate, magnitude and the kinetics of the aggregation process.

2.5.2. Determination of red blood cell deformability with three different methods

Erythrocyte deformability was determined in parallel by bulk filtrometry (Carat FT-1 filtrometer), slit-flow ektacytometry (RheoScan D-200) and rotational ektacytometry (LoRRca MaxSis Osmoscan).

2.5.2.1. Bulk filtrometry method

Carat FT-1 filtrometer (Carat Ltd., Hungary) was used for determining red blood cell deformability. The device is based on the St. George's blood filtrometer technique.

The tests need approximately 1.5 ml of blood. From the blood samples 5% red blood cell – phosphate buffered saline (PBS, osmolality: 295 ± 5 mOsm/kg, pH: 7.4) suspension aliquots were prepared and were being filtrated through a 5 μm pore-sized polycarbonate Nucleopore® filters (Whatman Co., UK) at constant filtration pressure (4 cmH2O). The unit interfaced to a computer which automatically analyzes the sequential flow rates by fotodetectors and calculates the initial relative filtration rate (IRFR) and the relative cell transit time (RCTT), according to the following formula: RCTT = [(IRFR-1 – 1)/Hct] + 1, where Hct is the hematocrit of the suspension. The RCTT value increases with the decrease of red blood cell deformability.

2.5.2.2. Slit-flow and rotational ektacytometry

For the ektacytometrial measurement of red blood cell deformabilty a Rheoscan D-200 slit-flow ektacytometer (Sewon Meditech Inc., Korea) and a LoRRca MaxSis Osmoscan rotational ektacytometer (Mechatronics BV, The Netherlands) were used.

For the measurements 5 μl of whole blood was taken into high-viscosity fluid suspension (polyvinylpyrrolidone, PVP 360 kDa, Sigma Aldrich, USA, dissolved in PBS. PVP-PBS suspension viscosity: 32.5-34.7 mPas, osmolality: 290-305 mOsm/kg, pH~7.3).

The measurement is based on the analysis of the diffracted laser images pattern from the elongated red blood cells against sher stress. By the method the elongation index (EI) is determined in the function of shear stress (SS [Pa]). For comparison EI-SS curves, EI values at 3 Pa, as well as maximal elongation index (EImax) and the shear stress at half EImax (SS1/2 [Pa]) were used by Lineweaver-Burk analysis.

The EI at 3 Pa, EImax values decreases and the SS1/2 value increases with the impairment of cell deformability.

2.5.3. Determination of erythrocyte membrane (mechanical) stability's changes

Filtration of the red blood cells by the spleen is based on their deformability alterations, including mechanical properties. Thus, in following-up splenic function using various spleen preservation techniques micro-rheological investigations can be informative.

In this study we aimed to investigate the red blood cell's membrane (mechanical) stability related to splenectomy (SE) and various spleen preserving operations.

The animals were followed-up for one and a half year. The red blood cell deformability and membrane stability were tested by rotational ektacytometry (LoRRca MaxSis Osmoscan rotational ektacytometer (Mechatronics BV, The Netherlands).

2.6. Determination of leukocyte antisedimentation rate (LAR)

Our previous experiments showed increased leukocyte antisedimentation rate (LAR) especially under hyposplenic-asplenic conditions. We aimed to determine the LAR and hematological-hemorheological changes caused by vaccination 1.5 years after splenectomy and in animals with different amounts of residual spleen tissue.

Methods:

Vaccination by RabigenMono (Virbac S.A.) and Vanguard Plus5 (RabigenMono, Virbac S.A.) was applied in 17 animals (sham operated contol n=3, splenectomy n=3, partial and subtotal spleen resection n=3/each, spleen-autotransplantation n=5) in the 16th postoperative month triggering immune response. Blood samples were collected for this investigation in the 6th, 9th, 18th postoperative months, and before and 1 week after vaccination.

LAR was calculated by Bogar's method, erythrocyte sedimentation rate and leukocyte count were analyzed, and blood-smears were made.

2.7. Statistical analysis for laboratory investigations

Data are presented as mean values with standard deviation (means ± S.D.). Besides analysing individual animals' data, for general intra-group analysis we used ANOVA tests (Bonferroni's or Dunn's post hoc tests). For inter-group comparisons at definitive time points of the follow-up period Student's t-test or Mann-Whitney rank sum tests were used, depending on the data distribution. A p value of <0.05 was considered to be significant.

2.8. Vessel-lumen filling technique with a special synthetic resin mixture for study of neo-microvascularisation of splenic autotransplants for morphological imaging examinations with micro-CT (Nano-SPECT/CT) technique

For investigation of neo-microvascularisation and morpho-functional capacity of spleen-autotransplants we developed a special vessel-lumen filling technique with synthetic resin displaying ultra-low viscosity, and defined CT-density for micro-CT examinations.

The microvascular connection between greater omentum and spleen-autotransplants were traced. Using this imaging method data on the 3D vascular morphology of the post-splenectomy liver was also obtained.

This new *ex-vivo* morphological imaging technique was introduced on 12th and 18th postoperative months of the experiment.

2.9. Comparison of three non-invasive imaging (nuclear medical techniques) methods for the visualization of splenic-autotransplants in large animal model

Scintigraphy can help to identify of the functional residual splenic tissue, separates spleen from other tissue types and allows quantitative characterization.

In present grant three methods were applied to detect the splenic autotransplants: non-specific colloid scintigraphy, specific scintigraphy and hybrid technique, in which SPECT was combined with CT imaging technique. All methods were based on the phagocytic function of the spleen.

In *non-specific method* the colloid is phagocytized by the cells of the reticuloendothelial system, therefore the activity appears in the Kupffer cells of the liver and in the phagocytes of the bone marrow, too.

Specific scintigraphy is more sensitive, based on the fact that damaged red blood cells (RBCs) are filtered from the circulation by the spleen.

The *hybrid technique* helps in localization of splenic tissue which is important to visualize the spleen autotransplants.

All the measurements were performed under general anaesthesia with intramuscular (i.m.) ketamine (10mg/bwkg, CP-Ketamin – ketamine hydrochloride 10%, Produlab Pharma B.V., Netherlands) and xylazine (1 mg/bwkg, CP-Xylazin – xylazine-hydrochloride, 2%, Produlab Pharma B.V., Netherlands) combination.

2.9.1. Non-specific colloid scintigraphy

In non-specific colloid scintigraphy (n=3) the animals received Tc99m-labeled sodium-phytate i.v. in 10MBq/bwkg dosage and 60 minutes later dynamic data acquisition was performed by Cardio-C γ-camera, from AP and lateral images that was followed by SPECT. The animals lay in supine position. It was performed 12 month after the surgical intervention.

2.9.2. Specific scintigraphy

In specific scintigraphy (n=4), in the 12^{th} postoperative month, first blood (anticoagulated with heparin) was withdrawn from the animals, stannous-pyrophosphate was added to the samples, red blood cells were labelled with Tc-99m and denatured by heat treatment (50 ^{o}C waterbath) then 80-120 MBq activity was given back into the circulation. 30 minutes later data acquisition started using dual-headed gamma camera (Anyscan S, Mediso, Hungary) from anterior and left lateral views that was followed by SPECT acquisition.

2.9.3. Hybrid technique

In hybrid technique (animal's number: n=8, investigation's number: n=14) after preparation of the animals (giving back autologous red blood cells denatured by stannous-pyrophosphate and heat treatment) and dynamic and SPECT acquisition with a CT scan was performed..

During data acquisition the two detectors of the gamma camera were positioned perpendicularly to allow simultaneous acquisition from lateral and anterior views. Dynamic acquisition started immediately after injecting the radiopharmaceutical, recording images from both views (into 128x128 frames, with 3.24 mm pixel size). Then SPECT acquisition followed in the same position of the animals (64 projections on 360° arc.) From the dynamic series parametric images of the change rate between the 10th and 30th min were calculated. Tomographic images reconstructed using OS-EM algorithm were attenuation-corrected using Chang's method, and the total uptakes of foci accumulating radiopharmaceutical were expressed as fractions of the injected dose.

The investigations were performed 6 month (AU-1,2), 15 month (AU-1,3,4,5,6,7,8) and 18 month (AU-1,3,4,5,6) after the spleen-autotransplantation.

2.10. Stem cell activity

At the indicated time periods investigation of stem cell activity in blood samples took place. We adopted culturing process known for mouse and human cells for canine ones to follow-up the mobilized progenitors in peripheral blood. We looked for the proper doses and combinations of citokines for growing canine progenitor cells.

Quantitative changes were studied at 1^{st}, 2^{nd} week and 5^{th}, 6^{th}, 6.5^{th}, 9^{th}, 12^{th} and 18^{th} month after operation by the help of the *primary cell cultures of blood* in 20-24 dogs. Kinetics of the quantative changes was different in certain groups and suggested activity of autotransplanted chips.

At 12^{th} and 18^{th} postoperative month the *autotransplanted spleen „chips"* were removed also for investigation of stem cell activity in the tissues.

2.10.1. Cellularity in spleen samples originated from spleen resection and autotransplanted spleen chips

Survived cells were studied in theese spleen samples. Cellularity calculated in a weight unit of the spleens indicates intensity of regeneration in comparison with healthy dog's samples. Living cells were caunted by the help of hemocytometer using tripan blue dye.

2.10.2. Soft-gel colony assays to study progenitor and stem cells in spleen samples originated from the resected and autotransplanted spleen chips of dogs

2.10.2.1. Evaluating myeloid progenitor cells

Spleen "chips" were removed sterile way from the dogs, cell suspensions are prepared by gentile pressing cells from them into 1 mL medium. Then medium with preserve-free heparin was used in 1:1 (v/v) ratio and single-cell suspensions were obtained by washing through narrow syringe needles. Ficoll-Iodamide gradient centrifugation separates mononuclear cell fraction in the "buffy coat", which was used for soft-gel cultures.

Mononuclear spleen cell suspensions in 5x106 and 106 cell/mL concentrations were used in soft-gel cultures. Methylcellulose in 1.2 % final concentration supported growing of progenitor cells. Modified McCoy's 5A medium with 20 % FBS and stimulatory factors, namely 300 ng/mL rh G-CSF, 100 ng/mL canine rc GM-CSF, 100 ng/mL canine rc SCF were used. These final concentrations of stimulatory factors were certified optimal for growing canine progenitor cells according to our preliminary studies. 3-3 cultures were used paralelly and they were incubated for 14 days in CO_2 incubator.

At the end of incubation period colonies were counted under stereo microscope. Colonies larger than 50-cells were counted. Morphology of cells in colonies were studied both *in situ* and cytospin preparations.

2.10.2.2. Evaluation of CFC-HPP (Colony-forming cells with a high proliferative potential)

In the spleen chips from autotransplanted dogs even more **primitive stem cells** were recognized. Besides 50 cell-containing colonies some very large cell-dense colonies were also seen, which contain more than 200 cells during the same 14 day-long incubation. This indicates a much higher proliferative potential and study their morphology showed more cell line in these colonies.

2.10.3. Evaluating Pre-B progenitor cells

Mononuclear cells obtaining by Ficoll gradient centrifugation were used from spleen samples. Cells were grown in Iscove Modified Dulbecco Medium in 106/mL concentration. In culturing bottles rh IL-7 helped to concentrate pre-B cells. After 4 days the nonadherent cells were harvested and this cell suspension was used for preparing soft gel methylcellulose cultures.

5x105 cells were inoculated, which was certified the best according to our preliminary studies. Using 30 % FBS and 30 ng/mL rh IL-7 we supported proliferation of pre B progenitor cells, which formed colonies during 14 days in CO_2 incubator.

At the end of incubation period colonies were counted under stereo microscope. Colonies larger than 50-cells were counted. Morphology of cells in colonies were studied both *in situ* and cytospin preparations.

2.10.4. Evaluation of dentritic cells

Mononuclear cells obtaining by Ficoll gradient centrifugation were used from spleen samples. Using IMDM medium cells in 3x105/mL, 5x106/mL and 106/mL concentrations were grown in culture flasks. After 2 hours incubation in 37 ^{o}C medium was removed with nonadherent cells and

the adherent cells were cultured further int he presence of rh IL-4 and rc canine GM-CSF in 20-2 ng/mL concentrations. LPS was also used to stimulate differentiation of dendritic cells.

After 7 days the adherent cells were studied under inverted microscope. Their colony formationand characteristic morphology of the cells were studied, too.

2.11. Histological investigations

After the extermination of the animals on the 12th, 15th and 18-19th postoperative months we got and prepared different tissue samples (spleen, splenic autotransplants (remnant spleen tissues), liver, kidney, small intestine, lung and heart) for complete light- and electron microscopic examinations.

2.11.1. Light-microscopic investigations

Histological analysis of HE stained sections of samples (residual and autotransplanted spleen, and liver, kidney, pancreas, small intestine, and lung) from recent and earlier experiments (OTKA K-04933, 2005-2009) has been completed. The collected tissue samples were formalin fixed and paraffin embedded according to the Departmental routine protocol.

Currently we are proceeding with further histochemical tools Prussian blue staining of siderophages has been completed. The appropriate samples have been selected and been processed: CD68 immunohistochemical labeling of macrophages.

4 μm sections were deparaffinated with xylol and 96% etanol, endogen peroxidase activity was blocked using 0,5% methanol containing hydrogen-peroxide for 30 minutes, washed with distillated water and stained for CD68 in Bond™ Max autostainer. Sections were pre-treated in Novocastra Bond Epitope Retrieval Solution. DS9800 Leica detection system was used for visualization.

Two criteria of histological evaluation were adopted: the qualitative and quantitative analysis. The qualitative analysis consisted of the evaluation of the fragments, with emphasis in the comparison with the normal splenic tissue being verified the capsule, the white pulp, the red pulp, the splenic tissue structure, and the vascularization. The quantitative analysis consisted of counting different cells (histiocytes, lymphocytes).

2.11.2. Electron-microscopic examinations

We prepared residual (partial or subtotal resected) spleen, splenic autotransplants- and liver samples for the electron-microscopic examinations.

Tissue blocks (not larger than 1 mm^3) were cut out from the liver and spleen, and put into a fixative containing 2% paraformaldehyde and 1% glutaraldehyde dissoleved in 0.1M phosphate buffer (pH: 7.4). After fixation, tissue blocks were washed several times in cacodylate buffer (pH: 7.4) then postfixed in 2% osmium tetroxide for 2 hours in room temperature. Following several washes in cacodylate buffer (pH: 7.4), tissue blocks were dehydrated and embedded into Durcupan ACM resin.

Ultrathin sections were cut, collected on Formvar-coated single-slot grids, and counterstained with uranyl acetate and lead citrate.

Sections were investigated with a JEOL 1010 transmission electron microscope and photographed with an Olympus Veleta CCD camera. Digitized images were processed with AdobePhotoshop CS5 sotware.

2.12. The effect of preventive medication on beagle's physiological parameters in long-term follow-up experiments: dental calculi removing, vaccination

The aim of this study was to compare the change in hematological parameters before and after different preventive procedures during the follow-up period.

In the 3rd postoperative month dental calculus was removed and gingivitis was treated.

Animals received yearly vaccination at 15th postoperative month.

Hematological measurements were performed before and after these procedures.

2.12.1. Dental calculi removing

Dental plaque is a biofilm or mass of bacteria that grows on surfaces within the mouth. A form of hardened dental plaque is calculus or tartar. Tartar can cause bad breath, receding gums, gingivitis, which chronic form can drive to periodontitis. Several data show a clear statistical link between gum disease and heart disease in dogs.

In our long-term follow-up splenic study comparing the effect of the splenectomy with different spleen preserving techniques (resection, utotransplantation) to prevent periodontitis we have removed the calculus in experimental animals.

The aim of our investigation was to detect the possible effect of tartar removing on physiological parameters.

Methods:

Dogs were anaesthetized on 4th postoperative month and we determined the dental calculus and gingivitis index. Calculus was removed with ultrasonic piezo scaler (Dental Woodpecker UDS-K Ultrasonic Piezo Scaler, Guilin Woodpecker Medical Instrument CO., LTD). Animal's mouth was rinsed with chlorhexidine-digluconate 0,2% w/v (Corsodyl, GlaxoSmithKLine, Brentford, UK) for two weeks.

Blood samples (for RBC [T/l], WBC [G/l], Hgb [g/dl], Neutrophils [G/l], Lymphocytes [G/l]) body weight, body temperature were decected before and three weeks after calculus removal.

The body weight was measured with KRUUSE PS 250 (Slimline, Denmark) digital platform scale.The body temperature was detected with digital thermometer Eco Temp Basic (OMRON, Japan).

2.12.2. Vaccination

For regular, yearly vaccination of the animals, 15 month after the operations, killed and adjuvanted rabies vaccines (RabigenMono, Virbac S.A.) and modified live canine distemper-adenovirus type 2-parainfluenza-parvovirus vaccine (Vanguard plus 5, Pfizer Animal Health S.A.) were given.

Blood samples were taken before the vaccination and after it on the 1st week fot the following examinations:, RBC [T/l], WBC [G/l], Hgb [g/dl], Neutrophils [G/l], Lymphocytes [G/l], Plt [G/l] Mo+Gr%, Lymph%, blood-sedimentation (We), leukocyte antisedimentation rate (LAR).

3. RESULTS

3.1. Changes of red blood cell aggregation parameters

Erythrocyte aggregation was determined in parallel by light-transmittance aggregometry (Myrenne MA-1 aggregometer) and syllectometry (LoRRca).

Results

Erythrocyte aggregation decreased three months after splenectomy, with lower aggregation index and elongated aggregation time. It was more or less associated with relatively lower hematocrit and fibrinogen concentration. However, in autotransplantated animals a relatively higher fibrinogen did not increase the aggregation markedly. Spleen resection resulted in the most controversial red blood cell aggregation findings, and it seems, that the degree of the resection is an influencing factor.

Conclusions

Splenectomy alters erythrocyte aggregation, spleen autotransplantation can be useful to preserve filtration function. However, the degree of restoration shows individual differences with a kind of „functional periodicity". Spleen resection controversially influences erythrocyte aggregation parameters. *The subtotal resection is supposed to be worse than spleen-autotransplantation.*

Publications:

1./ Kiss F., Furka I., Mikó I., Németh N.: ***An overview on red cell aggregation, as an important factor determining microcirculation. Links to the microsurgical research.*** In: Shinji Uemoto (Ed), ISEM2014 Abstract, Kyoto University, Japan, pp. 35-35., 2014.

2./ Kiss F; Tóth E; Pető K; Mikó I; Németh N: ***The investigation of interspecies diversity of erythrocyte aggregation properties by two different photometric methods in four animal species.*** Journal of Animal Physiology and Animal Nutrition (Berl) 99(6). 1074-1083., 2015.

3./ Mikó I., Kiss F, Furka I. Tóth E., Pető K., Furka A., Tóth L., Benkő I., Varga J., Németh N.: ***A vörösvérsejt-aggregációs paraméterek változása lép-autotranszplantációt és splenectomiát követően állatkísérletes modellen hosszú távú posztoperatív utánkövetéses vizsgálatok során.*** Magyar Sebészet 68(3). 133-133., 2015.

4./ Mikó I., Németh N., Pető K., Furka A., Tóth L., Furka I.: ***Changes of red blood cell aggregation parameters in a long-term follow-up of splenectomy, spleen-autotransplantation and partial or subtotal spleen resections in a canine model***. Clinical Hemorheology and Microcirculation 67. 91-100., DOI 10.3233/CH-170264 IOS Press, 2017.

3.2. Comparative erythrocyte deformability investigations by filtrometry, slit-flow and rotational ektacytometry

Erythrocyte deformability was determined in parallel by bulk filtrometry (Carat FT-1 filtrometer), slit-flow ektacytometry (RheoScan D-200) and rotational ektacytometry (LoRRca MaxSis Osmoscan).

Results

By filtrometry, relative cell transit time increased in the SE group (mostly in animal Nr. SE-3), showing the highest values on the 3rd, 9th and in 18th p.o. months. Elongation index values decreased in this group (both by slit-flow and rotational ektacytometers). In general, AU and two resection groups' values were lower versus control and higher than in SE.

Conclusions

Forasmuch in the circulation both elongation by shear stress and filtration occur, these various erythrocyte deformability testing methods together may describe better the alterations. Considering

the possible complications related to functional asplenic-hyposplenic conditions, *individual analysis of cases is highly important.*

Publications:

1./ Mikó I., Tóth E., Kiss F., Furka I., Furka A., Pető K., Németh N.: ***Comparative investigations for evaluating red blood cell deformability alterations related to splenectomy and various spleen-preserving operation types in a follow-up study, using filtrometry, slit-flow and rotational ektacytometry***, Biorheology 52(1-2). 154-155., 2015.

2./ Mikó I., Sógor V., Kiss F., Tóth E., Pető K., Furka A., Furka I.,Ványolos E., Tóth L., Varga J., Szigeti K., Németh N.: ***Vörösvértest-deformabilitás meghatározása filtrometriával, slit-flow és rotációs ektacytometriával splenectomiát, lépresectiót és autótransplantációt követően.*** Érbetegségek XXIII (2). 40-40., 2016.

3./ Mikó I., Németh N., Sógor V., Kiss F., Tóth E., Pető K., Furka A.,Ványolos E., Tóth L.,Varga J., Szigeti K., Benkő I., Oláh VA, Furka I.: ***Comparative erythrocyte deformability investigations by filtrometry, slit-flow and rotational ektacytometry in a long-term follow-up animal study on splenectomy and different spleen preserving operative techniques: Partial or subtotal spleen resection and spleen autotransplantation.*** Clinical Hemorheology and Microcirculation 66:(1), 83-96., 2017.

3.3. Erythrocyte membrane (mechanical) stability changes in splenectomy and related to various spleen-preserving operation types in a long-term follow-up animal study

Results

Although deformability values showed fluctuating differences among groups, mechanical stability values alone didn't show significant difference over the follow-up period. However, the SE group expressed the largest deterioration in elongation index values against the mechanical stress applied. This worsening was the most obvious in the 3rd postoperative month. When we analyzed the cases individually, one splenectomized animal markedly expressed impaired deformability and mechanical stability in the 3rd and 9th month.

Conclusions

We concluded, that erythrocyte membrane stability test can be a *useful supplementary tool for enforcing micro-rheological alterations* when following-up splenic function.

Publication:

1./ Mikó I., Sógor V., Tóth E., Kiss F., Furka I., Furka A., Oláh V. A., Pető K., Németh N.: ***Vörösvértest mechanikai stabilitásváltozások splenectomia és különböző lépmegtartó beavatkozások kapcsán hosszú távú utánkövetéses állatmodellben.***, Érbetegségek XXIII (2). 41-41., 2016.

Preparation of relevant scientific publication(s) presenting our findings is underway.
We do not tend to submit details till the acceptance of article.

3.4. Change of leukocyte antisedimentation rate with splenectomy and different amount of residual spleen after the mandatory vaccination

Results

After vaccination control and partial resection (one-third resection) groups showed the highest leukocyte antisedimentation rate decrease.

In the spleen-autotransplantation group, the leukocyte antisedimentation rate were almost equal while in splenectomy and subtotal spleen resection group a greater leukocyte antisedimentation rate increase were observed. Leukocyte count did not change.

The leukocyte antisedimentation rate increased in the subtotal spleen resection (two-third resection) group. Smears showed numerous stab-segmented neutrophils and altered-shape erythrocytes.

Conclusions

In long-term follow-up animal study the leukocyte antisedimentation rate (LAR) can be an applicable and important parameter for monitoring/assessing the residual spleen tissue's function. In case of asplenic conditions, the leukocyte antisedimentation rate could be particularly important to signal the possibility of potential complications in time.

However, we emphasize that to form an opinion, other laboratory measurements have to be taken into consideration case by case.

Publications:

1./ Mikó I., Tóth E., Kiss F., Furka I., Pető K., Furka A., Deák Á., Ványolos E., Oláh A., Tóth L., Benkő I., Németh N.: ***Change of leukocyte antisedimentation rate and other hematological and hemorheological parameters in beagle dogs with splenectomy and different amount of residual spleen after the mandatory vaccination.*** European Surgical Research 55(1). 158-159., 2015.

2./ Mikó I., Tóth E., Kiss F., Furka I., Pető K., Furka A., Deák Á., Ványolos E., Tóth L., Benkő I., Németh N.: ***A leukocyta-antiszedimentációs ráta és egyes hematológiai, haemorrheologiai paraméterek változásai a beagle kutyák kötelező védőoltását követően lépeltávolító, valamint különböző típusu lépmegtartó műtétek után.*** Magyar Sebészet 68. 133-134., 2015.

Preparation of relevant scientific publication(s) presenting our more findings is underway.
We do not tend to submit details till the acceptance of article.

3.5. Vessel-lumen filling technique with a special synthetic resin mixture for study of neo-microvascularisation of splenic autotransplants for morphological imaging examinations with micro-CT technique

Results

Using this imaging method data on the 3D vascular morphology of the post-splenectomy liver was also obtained.

Conclusions

The combined technique was suitable studying asplenic-hyposplenic condition. The experimental data *may contribute to follow-up protocol of the future spleen-autotransplanted patients after posttraumatic splenectomy to identify the important splenic functions.*

Publications:

1./ Kiss M., Nemeskéri Á., Kürti Zs., Gáti E., Dorogi B., Dudás I., Furka I., Németh N., Furka A., Mikó I.: ***Új érlumen-feltöltési technika, autotranszplantált lépdarabok neovascularisatiójának tanulmányozása***. Magyar Sebészet 66. 92-92., 2013.

2./ Szigeti K., Máthé D., Horváth I., Kiss M., Karlinger K., Nemeskéri Á., Furka I., Mikó I.: ***Microvascularisatio ex vivo vizsgálata nagy felbontású CT és MRI segítségével***. Magyar Sebészet 66. 109-109., 2013.

3./ Kiss M., Nemeskéri Á., Kürti Zs., Gáti E., Dorogi B., Dudás I., Furka I., Németh N., Pető K., Ványolos E., Furka A., Mikó I.: ***New Vessel-Lumen Filling Technique, for the Study of Neo-Microvascularisation of Autotransplanted "spleen chips".*** In: Shinji Uemoto (Ed), ISEM2014 Abstract, Kyoto University, Japan, pp. 55-55., 2014.

4./ Kiss M., Nemeskéri Á., Kürti Zs., Gáti E., Dorogi B., Dudás I., Furka I., Németh N., Pető K., Ványolos E., Furka A., Mikó I.: ***New Vessel-Lumen Filling Technique, for the Study of Neo-Microvascularisation of Autotransplanted "Spleen Chips"***. European Surgical Research 52. 156-156., 2014.

Preparation of relevant scientific publication(s) presenting our more findings is underway.
We do not tend to submit details till the acceptance of article.

3.6. Comparison of three non-invasing imaging (nuclear medical techniques) methods for the visualization of splenic-autotransplants in large animal model

Results

3.6. 1. Non-specific colloid scintigraphy

With colloid scintigraphy the highest activity was found in the liver. At the expected site of the splenic-autotransplants some focal activity accumulation was detected, but the autotransplants were poorly detectable.

3.6.2. Specific scintigraphy

By spleen-specific scintigraphy on the parametric images only the spleen and the urinary bladder were visualized, proving the specificity of the method. Most of the spleen-autotransplants were detectable, although not 100% of them and varying uptake values were found.

3.6.3. Hybrid SPECT-CT technique

Using hybrid SPECT-CT, the identification of the splenic-autotransplants was easier and more precise, although still not all of them were seen. Scintigraphy demonstrated the function of preserved splenic tissue in all cases, but not all replanted chips were found at all examined time points. A possible explanation of that is the temporary hypofunction of the autotransplants. Histological and laboratory examinations indicated hyposplenic condition in these cases, supporting this theory. In some animals the liver curves also showed a monotonous increase in time, indicating that part of Tc-99m released from the RBCs; yet the spleen function could be judged.
In the 12th postoperative month 13 scintigraphic examinations were performed in all groups combined, but because of technical reasons the findings were inadequate for the quantitative evaluation of the residual spleen tissue function.
In the 15th and 18-19th postoperative months further 21 examinations were carried out. From the 10 scintigraphic studies that were performed on animals with intact spleen or part of it, 7 showed normal spleen and liver time-activity curves. 3 animals (two R2/3 and one R1/3) presented normal (i.e. increasing) splenic curves, but their liver curves also showed an increase, which leads to the suggestion that part of Tc-99m released from the RBCs; yet the spleen function could be judged.
Altogether 35 examinations were performed, but not all of them were successful, partly because of technical problems (eg. artefacts originated from movements of the animal), partly in result of anaemia of the animals (there were not enough red blood cells to bind).

Conclusion for the three methods

While **non-specific colloid scintigraphy** was somewhat unreliable and unsatisfactory, the spleen-specific techniques had proved to be more successful in visualization of functioning splenic tissue.
By **spleen-specific scintigraphy** most of the spleen-autotransplants could be visualized, with varying uptake values. The planar parametric images indicated functioning splenic tissue in all animals.
Using **hybrid SPECT-CT**, *the identification of the splenic-autotransplants was easier and more precise*, although not all of them were seen. Histological examinations indicated hyposplenic condition in these cases.
The hybrid SPECT-CT imaging, with CT for localization of the spleen-autotransplanted chips, *establishes the detection of functioning spleen tissue with higher confidence than SPECT in itself*.

Nevertheless, even CT scan was unable to indicate every autotransplanted chips that the subsequent operative follow-up (operative intervention, micro-CT) revealed. No significant relation was found between the uptake of the radiopharmaceutical and the percentage of the preserved spleen tissue. In the future our aim is to find and analyse the correlation between the number of functioning auto transplanted spleen chips and the distribution of the radiopharmaceutical in the tissues.

Publications:

1./ Mikó I., Pető K., Furka A., Németh N., Kiss F., Kalóczkai G., Deák Á., Nagy T., Furka I., Galuska L., Varga J.: ***Scintgraphic method to prove the survival of autotransplanted splenic tissue in canin model. A pilot study.*** European Surgical Research 52. 241-241., 2014.

2./ Mikó I.; Pető K.; Furka A.; Németh N.; Kiss F.; Kalóczkai G.; Nagy T.; Galuska L.; Varga J.: ***Maradék lépszövet kimutatása SPECT-CT vizsgálattal állatkísérletes modellben különböző típusú lépmegtartó műtéteket követően.*** Magyar Sebészet 67. 189-189., 2014.

3./ Mikó I., Pető K., Furka A., Furka I., Németh N., Ványolos E., Garai I., Barna S., Nagy T. Kiss F., Deák Á., Kalóczkay G., Balogh D., Varga J.: ***Működő lépszövet kimutatása SPECT-CT vizsgálattal szervmegtartó műtéteket követően állatmodellben***. Magyar Sebészet 68(3). 123-123., 2015.

4./ Mikó I., Pető K., Furka A., Németh N., Ványolos E., Garai I., Barna S., Deák A., Kiss F., Nagy T., Balogh D., Furka I., Varga J.: ***Noninvasive monitoring of functioning spleen tissue after organ saving operations in animal model***. European Surgical Research 55(1). 158-158., 2015.

5./ Mikó I., Pető K., Deák Á., Németh N., Ványolos E., Furka A., Furka I., Nagy T., Garai I., Galuska L., Varga J.: ***Comparison of three imaging methods for the visualization of splenic-autotransplants in large animal model.*** European Surgical Research 57(Suppl.1). 122-122., 2016.

6./ Mikó I., Pető K., Deák Á., Németh N,. Ványolos E., Furka A., Furka I., Nagy T., Garai I., Galuska L., Szigeti K., VargaJ.: ***Különböző leképezési módszerek hatékonyságának összehasonlítása lép-autotraszplantátumok kimutatásában állatmodellen.***, In: Program of 63rd Congress of Hungarian Surgical Society, pp. 14-14., 2016.

Preparation of relevant scientific publication(s) presenting our summarised findings is underway. We do not tend to submit details till the acceptance of article.

3.7. Histological investigations

3.7.1. The light-microscopic investigations

Splenectomy group

Analysis of the liver: Generally the histological structure of the liver seems to be intact. Hepatocytes look normal. Hepatic sinuses seem to be wider than normal. Kupffer cells' number increased and seem to be hypertrophized. In one animal, in the portal tracts and in sinusoids there are mild granulocytic infiltration.

In the case of splenectomy liver was heavily affected in every animal. The number of Kupffer cells was increased. In one case there was a mild neutrophil leukocytic infiltration in the portal fields and in the sinusoids.

Analysis of the lung: In one case, there was a moderate neutrophil leukocytic infiltration in the interstitium of the lungs. At another animal there were moderate signs of incipient pneumonia in small foci.

Spleen autotransplanted group

Analysis of the autotransplantated spleens: Splenic capsule sometimes normal, other times with hyalinization and sometimes severe fibrosis. The white pulp was preserved. Sometimes is a great

depletion of the white and red pulp. Were found many activated macrophages, with repleted cytoplasm of hemosiderine granules.
Analysis of the liver: Generally, the sinusoids are wider, stagnant and hemosiderin can be seen. In one case we saw a lot of siderophags and increased number of Kupffer-cells. In two cases there was an incipient neutrophil leukocytic infiltration in the portal fields.
Generally the histological structure of the liver seems to be intact. Hepatocytes look normal. In some cases the sinusoidal structure of the liver seems to be wider than normal. In one experimentel animal the Kupffer cells' number are increased and seem to be hypertrophized, in the cytoplasm with siderophages (functional activity of the Kupffer cells). In two cases, in the portal areas we detected neutrophyl leukocytic infiltration.
Analysis of the lung: In four cases there were lung lesions. In small foci there were neutrophil leukocytic infiltration. In four animals we detected a moderate interstitial leukocytic infiltration.

Spleen resection groups
Analysis of the remnant spleens: The residual spleen was normal with diverse stagnation. In some cases the white pulp was moderetaly withered.
Analysis of the liver: There were diverese Kupffer cell proliferation in the liver with diverse stagnation. In some cases there was a neutrophil infiltration in the sinusoids and in the periportal fields. In these animals we also found neutrophil infiltration *in the lungs* and the white pulp of the spleen was withered.

Conclusion
Interference with the physiological functions of the spleen may reduce the latter's efficacy which may result in increased susceptibility towards infections. This might explain the occurrence of neutrophilic infiltrates in the interstitial compartment of the liver and lungs.

3.7.2. Electron-microscopic examinations

Splenectomy group
Analysis of the liver: The histological structure of the liver seems to be intact. Hepatocytes look normal. However, there are cells scattered among the hepatocytes which present electro-dense cytoplasm and even denser inclusions looking like phagosomes. Hepatic sinuses seem to be wider than normal. Kupffer cells seem to be hypertrophized and also contain electro-dense inclusions.

Spleen autotransplanted group
Analysis of the autotransplantated spleens: the histological structure generally does not seem to be altered, but some cases there are narrow arborizing connective tissue fiber (??) bundles with a diameter of less than 1 μm in some areas of the parenchyme. A continuous electro-dense precipitation occurs on the surface of the bundles. An interesting cell population with electro-dense or electro-lucent cytoplasm can be observed in the parenchyme. These cells accumulate numerous inclusions with inhomogeneous content. In addition, blood vessels the wall of which is lined by cuboidal epithelium can also be frequently observed.
Analysis of the liver: At the border of the hepatic lobule and the perilobular connective tissue, cells with multivesicular bodies appear in substantial numbers. The multivesicular bodies may reach the size of 0.5 μm.

Some hepatocytes present electro-dense others electro-lucent cytoplasm. The two populations are not segregated from each other and can be observed in more-or-less the same numbers. Inclusions with inhomogeneous content frequently appear in both types of hepatocytes. Some of the sinosoids seem to be enlarged.

Spleen resection groups

Partial (one-third) spleen resection

Analysis of the remnant spleens: The cell density in the splenic cords seems to be higher than normal. Otherwise the substance of the spleen appears to be intact.

Analysis of the liver: Inclusions with inhomogeneous content appear in a few hepatocytes. Otherwise the histological structure of the liver seems to be intact.

Subtotal (two-third) spleen resection

Analysis of the remnant spleens: Reticular cells can be seen in large numbers both in the white and red pulps. On the other hand, however, lymphocytes and other white blood cells can only be occasionally found. Blood vessels lined by cuboidal cells can be observed.

Analysis of the liver: The histological structure of the liver seems to be intact.

Preparation of relevant scientific publication(s) presenting our summarized findings is underway. We do not tend to submit details till the acceptance of article.

3.8. Stem cell activity

3.8.1. Cellularity in spleen samples originated from spleen resection and autotransplanted spleen chips

Results

Cellularity of spleen was significantly higher in autotransplantated chips. This suggests that there is a regeneration in cell lines by the help of the stem cells.

We studied whether stem cell populations with characteristic features are detected in these autotransplanted spleen chips inserted in greater omentum.

We found an *expanded granulocyta-macrophage progenitor cell pool and significantly more matured phagocytes.*

Earlier *stem cells* are also detected in these autotransplanted spleens. These CFC-HPP, Colony-forming cells with a high proliferative potential (CFC-HPP) were seen only in the soft-gel cultures of autotransplanted and not in cultures originated from spleens resected in their physiological place. This shows very intensive regeneration in autotransplanted spleen chips.

Regeneration of hemopoietic progenitor cell population were less intensive in dogs *with partial spleen resection.*

Cellularity of spleens of dogs *with partial spleen resection was significantly higher* than in control sham operated dogs *but less than in autotransplanted spleen chips.* It may be surprising that there were no difference in cellularity between dogs with subtotal resection and spleens of sham operated controls.

Conclusions

We studied whether stem cells with proper functions are found in the autotransplanted spleen chips. Spleen has important function as an organ of immune system. Splenectomy results in serious disorder in elimination of pathogens entering from gastrointestinal system and septic shock may develop. We looked for stem cells which may regenerate immune cell populations in the spleen samples. These stem cells are not able to be distinguished from each others or the matured cells in the mononuclear cell fraction. Special functional tests are required and soft-gel colony assays were used to study progenitor and stem cells in spleen samples.

3.8.2. Evaluating myeloid progenitor cells

Results

In the special colony assay to study **myeloid progenitor cells**, granulocyte-macrophage progenitor cells (CFU-GM) were measured, which produce phagocytes and have a great role in natural immune responses. Each samples from *autotransplanted spleen chips* includes CFU-GM progenitors with proper functions. Their colony forming capability maintained their own populations and the matured phagocytes in the cell cultured jointly showed their stem cell feature. These CFU-GM progenitors appeared in autotransplanted spleens as early than 1 months after operation. In addition marked expansion of CFU-GM pool was seen, which *indicated intensive regeneration.* In these *autotransplanted spleen chips were 2 times more CFU-GM cells than in* spleens of *sham operated dogs* in the same weight units.

Conclusions

This showed that these *autotransplanted spleen chips* found proper conditions in greater omentum to *survive and preserve their function in immune responses.*

In spleen samples from dogs with *partial splenectomy* we found also intensive regeneration. Significantly higher numbers of CFU-GM were found than in sham operated controls calculated for weight units, however these values were significantly less than in autotransplanted spleen chips.

3.8.3. Evaluation of CFC-HPP (Colony-forming cells with a high proliferative potential)

Results

The most intensive regeneration was seen in autotransplanted samples. Even more **primitive stem cells** became detectable in these samples. These are the HPP-CFC (colony forming cells with a high proliferative potential) stem cells. These have much greater proliferative capacity than the progenitor cells and during the same culturing period these HPP-CFC stem cells produce several hundreds - thousands descendant cells in comparison with CFU-GM progenitors which produced 50 to 100 cells. In some cultures we have seen these huge colonies even perceptible to the naked eyes. Ratio of these primitive stem cells is very low in normal tissues. That is why we do not see them in bone marrow or spleen samples of healthy animals.

Conclusion

Their appearance in our autotransplanted spleen samples indicates very intensive regeneration. We were not able to detect them in spleen samples originated from the dogs with partial or subtotal resection or in sham-operated controls.

3.8.4. Evaluating Pre-B progenitor cells

Spleen has an importance not only in nonspecific but also in specific immune responses. Especially B lymphocytes in spleen help to prevent infections by immunoglobulin production and protect against bacterial or other pathogen invasion from gastrointestinal system. Thus we studied **pre-B cell** population in spleen samples of dogs.

Results

1 month after operation we do not find pre-B cells in autotransplanted spleen chips. Regeneration of the population of pre-B cells was very slow. Even 8 months later there were significantly less pre-B cells in autotransplanted spleen chips than in sham-operated controls.

Conclusion

These findings correlated well our previous observations in mice. CD19+ B cells were significantly less in the blood of the mice with autotransplanted spleen chips than normal values after 8 months of operation (Transpl Immunol. 2006 Aug;16(2):99-104.).

3.8.5. Evaluation of dentritic cells

By studying circulating progenitor cells in blood we looked for connection between spleen and bone marrow during regeneration of spleen.

Results

In healthy animals numbers of circulating stem cells are very low. We found 9-10 progenitors/ 10^6 mononuclear cells in blood of sham-operated dogs.

Injury of spleen meant a great stimulus to mobilize stem cells from bone marrow. In blood of dogs with **partial resection** *16 times more stem cells* were found and in blood of dogs with **autotransplanted spleen chips** there were *20 times more stem cells.*

Conclusion

In our experiments we have seen that *bone marrow supported spleen regeneration during long period. Circulating bone marrow derived stem cells were significantly more than in controls in the blood samples even in 9^{th}, 12^{th} and 18^{th} postoperative months.*

Discussion

Our results may be useful for clinical practice as we used dogs, in which spleen functions are much similar to human ones than in mice.

Spleen is very important immune organ, which prevents spread of pathogens from gastrointestinal system. It has complex immune functions which have to be preserved as much as it is possible.

If we have to remove spleen we have to preserve healthy parts of spleen as much as it is possible.

Very important finding of our experiments is that **autotransplanted spleen chips have functions,** *contain several different types of stem cells which regenerate immune cell lines by producing mature immune cells. Their immune functions may be regenerated.*

In dogs with **subtotal spleen resection** we have seen **much less regeneration** *especially in CFU-GM progenitor cell population.*

In comparison we have seen a very intensive regeneration during long period and we found 2 times more CFU-GM progenitors in autotransplanted spleen.

Publication

1./ Mikó I., Németh N., Furka I.:: ***Spleen Autotransplantation in Mice***, In: Huifang Chen, Shiguang Qian (Eds) Experimental Organ Transplantation. Hauppauge (NY): Nova Science Publishers, pp. 107-120., 2013.

Preparation of relevant scientific publication(s) presenting our summarized findings is underway. We do not tend to submit details till the acceptance of article.

3.9. The effect of preventive medication on beagle's physiological parameters in long-term follow-up experiments: dental calculi removing, vaccination

Results

The tartar removal did not change the physiological parameters as haematological parameters (WBC, RBC, EOS, LYMPH), body weight and body temperature significantly.

BUT: We have found significant changes compared with control and splenectomised groups' data, also we detect intragroup change compared to the base data, before and after interventions.

Conclusions

In long-term follow-up research to improve the animal welfare and to prevent the possible complications, we warmly recommend the removing of dental tartar regularly.

The well-timed treatments and preventive interventions in our long-term follow-up study, did not affect significantly the outcome of the experiment. However the haematological and physiological parameters vary depending on type of surgical procedure and immunological state of animals.

Publications

1./ Deák Á., Tóth E., Pető K., Németh N., Ványolos E., Furka I., Mikó I.: ***Does tartar removal affect the physiological parameters of beagle dogs in long-term follow-up research?*** In: CEELA-2015 Proceeding, pp. 56-56. International CEELA III. Triannual Conference of the Hungarian Laboratory Animal Science Association and the Standard Committee, 2015.

2./ Deák Á., Tóth E., Németh N., Ványolos E., Furka I., Mikó I.: ***Does tartar removal affect the physiological parameters of beagle dogs in long-term follow-up research?*** European Surgical Research 57(Suppl.1). 96-96., DOI: 10. 1159/446131, 2016.

Preparation of relevant scientific publication presenting our findings is underway.

4. FINAL SUMMARY

Our aim was to monitor with different methods the long term state of the splenectomised, the spleen resected and spleen autotransplanted subjects due to splenic trauma.

Comparative investigations on beagle dogs could to indicate some predictive laboratory parameters/markers, signs of possible Overwhelming Postsplenectomy Infection(OPSI) or/and Disseminated Intravascularis Coagulation(DIC) syndromes based on hyposplenic-asplenic state after traumatic spleen injury operation with splenectomy or splenic sparing surgery and/or the application of new morphological imaging methods.

We wanted to call the attention to these signs of possible complications of hyposplenic/asplenic states.

1./ We could to monitor the loss or restoration of splenic filtration/immune-functions with some laboratory methods, e.g. microrheology, IgM, lipid profile, platelet functions' investigations.
In the future, during the micro-rheological analyses special attention is needed when comparing partial or subtotal resection groups and splenic autotransplantation groups.

2./ We introduced non-invasive direct/indirect nuclear medicine methods (planar gamma camera, SPECT) and proposed how to detect the functioning splenic tissue for investigation of spleen autotransplanted patients, and how to determine the quantity of functioning splenic tissue after spleen-autotransplantation and various spleen-resection techniques.

3./ The supplementary light and electronmicroscopic investigations together with immune-marking method could signe also the consequences of hypo/asplenic state on liver and lung but not in kidney, bowel, pancreas and heart

4./ We introduced a new morphological imaging method with an injection of a new special mixture of synthetic resin into the vascular system of abdominal organs for the visualization of the high-contrast polymerized casting material in the remnant splenic tissue' vasculature, the autotransplanted splenic tissue and the liver using high resolution scanning micro-SPECT/CT.
The statistical analysis of the vascular system's geometric parameters strengthened the functioning splenis tissue in the autotransplants.

5./ Investigating the spleen-regeneration -for the presence of hemo/lymphopoietic stem cells- we showed they can restore the autotransplants' function. Cellularity of spleen was significantly higher in autotransplantated chips. This suggests that there is a regeneration in cell lines by the help of the stem cells. Very important finding of our experiments is that autotransplanted spleen chips have functions, contain several different types of stem cells which regenerate immune cell lines by producing mature immune cells. Their immune functions may be regenerated.

We proposed to do parallel hematological, hemostaseological, micro-rheological tests, hybrid nuclear medicine imaging (e.g., SPECT/CT, microCT), stem-cell-, and histological investigations in a wide collaborative examinations for revealing the general and individual changes as well.

Very important to know, that loss of splenic function (asplenia by splenectomy or decrease in function (hyposplenia by partial or subtotal resection or autotransplantation) means a condition, but the magnitude of changes may also show individual alterations

The individual analysis of the data comparing with other parameters is very important.

We belive that our results may be useful for clinical practice as we used dogs, in which spleen functions are much similar to human ones than in mice.

5. PUBLICATION ACTIVITY

2013.

2 presentations on the 24th Congress of the Experimental Surgical Section of the Hungarian Surgical Society, 1 publication and 2 reviewed abstracts in Hungarian in the Hungarian Journal of Surgery. One abstract was submitted for the 12th Congress of the International Society for Experimental Microsurgery (ISEM), (Kyoto, Japan); and 3 abstract submissions are in progress for 49th Congress of European Society for Surgical Research (ESSR) that will be held in Budapest, where we are going to present new methods/techniques developed by us to detect the microcirculation of the autotransplanted spleen-chips.

One-one paper has been published in Hungarian Journal of Surgery and in a scientific book titled Experimental Organ Transplantation. Hauppauge (NY): Nova Science Publishers.

2014.

1 poster presentation on 62th Congress of the Hungarian Surgical Society, with 1 reviewed abstracts in the Hungarian Journal of Surgery. 1 oral and 1 poster presentation were on the 49th Congress of European Society for Surgical Research (ESSR) with 2 reviewed abstracts in the European Surgical Research. One oral presentation was on the 12th Congress of the International Society for Experimental Microsurgery (ISEM) in Kyoto/Japan.

2015.

6 presentations were in different conferences. Two-two posters were presented at the 15th International Congress of Biorheology and 8th International Congress of Hemorheology in Seoul/South-Korea, at Congress of Hungarian Society of Hemorheology and at 25th Experimental Surgical Congress of Hungarian Surgical Society and at the 50th Jubilary Congress of European Society for Surgical Research (ESSR) in Liverpool.

Two-two peer-reviewed abstracts have been published in Biorheology, in Hungarian Journal of Surgery and in the European Surgical Research. One paper has been published in Journal of Animal Physiology and Animal Nutrition.

2016.

5 presentations were in different conferences. Two poster presentations were at the 51st Congress of European Society for Surgical Research (ESSR) with two peer-reviewed abstracts in the European Surgical Research and two oral presentations were at the Congress of Hungarian Hemorheological Society in Balatonkenese with 2 peer-reviewed abstracts in the Scientific Journal of the Hungarian Soceity for Angiology and Vascular Surgery (Érbetegségek). 1 oral presentation was at 63th Congress of the Hungarian Surgical Society in Budapest. Two papers were submitted.

2017.

Two papers have been published in Clinical Hemorheology and Microcirculation.

Under submission are the next papers:

1./ Mikó I., Tóth L., Furka A., Ványolos E, Tóth E., Németh N., Antal M., Furka I.:
"Complex histopathology of remnant splenic, hepatic and pulmonary tissues may highlight the possible functional hyposplenic or asplenic condition due to spleen preserving surgery - veterinary model data".

2./ Mikó I., Furka I., Kiss M., Dorogi B., Szuák A., Nemeskéri Á., Németh N., Pető K., Furka A., Ványolos E., Trencsényi Gy., Garai I., Tóth L., Máthé D., Horváth I., Szigeti K.:
"Vessel-lumen filling technique with a special mixture of synthetic resin for study of neo-microvascularisation of splenic autotransplants. Morphological imaging examinations with micro-CT technique".

3./ Mikó I., Varga J., Pető K., Deák Á., Németh N., Ványolos E., Furka A., Nagy T., Barna S., Garai I., Galuska L., Trencsényi Gy., Szigeti K., Tóth L., Kalóczkai G., Balogh D., Sajtos E., Kiss F., Furka I.:
"Comparison of three imaging methods for the visualization of the splenic-autotransplants in a large animal model".

4./ Mikó I., Deák Á., Tóth E., Németh N., Ványolos E., Furka I.,
"The effect of preventive medication on beagle's physiological parameters in long-term follow-up experiments: vaccination, dental calculi removing and antihelmintic treatment".

5./ Mikó I., Oláh VA, Tóth E., Furka A., Németh N., Ványolos E., Deák Á., Furka I.:
„Laboratory parameteters can predict asplenic/hyposplenic status after splenectomy and/or different spleen sparing surgical procedures: Hemato-hemostaseological, lipid and enzym levels's investigation (follow-up) in large animal experimental model".

6./ Mikó I. Benkő I., Furka A., Ványolos E., Furka I.:
„Stem cell activity after splenectomy, partial or subtotal spleen resection and spleen-autotransplantation. Comparative data in a large animal experimental model".

6. Ph.D. DEFENDE

On May 27, 2014 from the results of previous spleen-research project Erika Sajtos, M.D. defended her Ph.D. thesis entitled: "Follow-up possibilities with hemorheological measurements and imaging techniques in asplenic/hyposplenic conditions related to spleen-preserving surgical techniques in animal expiermental model". Supervisors: Iren Mikó and István Furka -senior researchers of this OTKA.

.

7. PERSONAL CHANGING

2013.
Since the start of the OTKA research Assistant Professor **Katalin Pető, M.D., Ph.D.** from 1st January 2013, **Ádám Deák, D.V.M., Ph.D.**, from 1st September, 2013 joined the OTKA as senior participants, abovementioned **Gergely Kalóczkai, M.D. full-time Ph.D. student** also joined as young researcher. Zoltán Klárik, young researcher changed jobs.

2014.

From January 1, 2014 Associate Professor **Ildikó Garai, M.D., Ph.D.,** medical director of ScanoMed and Assistant Professor **Anna Oláh, M.D., Ph.D.,** senior research fellow of the Department of Laboratory Medicine have joined as senior researcher.

2015.

From 1st September Ferenc Kiss, M.D., Ph.D., and Gergely Kalóczkai full-time Ph.D. student also changed jobs.
From 1st October **Viktória Sógor full-time Ph.D. student** joined as young researcher.

2016.

Assistant Professor **György Trencsényi, Ph.D.,** also joined as senior researcher.

20th May, 2018. Debrecen,

Prof. Irén Mikó, M.D., C.Sc., Ph.D.