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

Investigation of microvascular anastomosis regeneration in experimental surgical models

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1 INTRODUCTION

Microvascular anastomoses are used in many surgical specialties, particularly in reconstructive and transplant surgery. Many conditions must be met for proper vascular regeneration, so extra care is particularly important when restoring blood supply. Tissue preparation, preparation, adequate wetting, the degree of trauma that has occurred or is occurring, inadequate removal of adventitia, mismatch of the angle, twisting of the vessel, haemorrhagic complications, thrombosis, inflammation and ischaemia-reperfusion injury, can all affect the success of anastomosis. When dealing with sensitive tissues, hydration of the surgical site with body temperature physiological saline is essential. However, there are not many objective studies on this, but rather empirical observations.

Hydration plays a critical role in preserving tissue viability, improving visualisation and reducing tissue trauma, especially in delicate microsurgical operations. The hydration status of tissues is crucial in the perioperative period during the preparation of microvascular anastomoses, as dehydration can lead to significant damage. Cellular damage from tissue dehydration can impair viability and raise the possibility of necrosis. In addition, dehydration can cause vasospasm, which complicates the anastomosis process and potentially impedes postoperative blood flow. Dehydrated tissues become brittle and lose elasticity, increasing the risk of accidental injury during suturing or manipulation. Dehydrated tissues also impair visibility by allowing blood and tissue debris to adhere to the surgical site, contaminating the area and making the procedure more difficult. Furthermore, dried blood vessels are more prone to endothelial damage, which increases the risk of thrombus formation, postoperative thrombosis and graft failure. Lastly, impaired healing due to perioperative dehydration affects cell migration, proliferation and differentiation, which are essential processes in wound healing.

Proper tissue hydration not only helps maintain tissue viability, but also facilitates the manipulation and suturing of delicate structures during microsurgical procedures. Neglecting tissue hydration during surgery can lead to increased complications, such as prolonged recovery time, higher rates of postoperative complications and poorer surgical outcomes. Despite its surgical importance, there is limited literature on tissue hydration, especially in terms of objective data such as histological, biomechanical and fluid dynamic observations.

Adequate regeneration of the anastomotic vessel is critical for restoring physiological vascular function. The process is similar to wound healing, with phases of inflamatory, proliferation and maturation. The infiltration of extracellular matrix-producing cells and the formation of a new matrix largely determine the biomechanical stability of the anastomosis. Ongoing efforts have been made to support regeneration processes and improve clinical

outcomes, for example by reducing operative time in sutureless techniques and by reducing foreign material through the use of tissue adhesives. Several drugs have been investigated for tissue regeneration, such as PACAP and BGP-15, which are promising drug candidates.

The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) may help in the healing process by reducing inflammation, apoptosis and oxidative stress. BGP-15 is a drug candidate molecule discovered by Hungarian researchers in the study of heat shock proteins and has additional protective effects in addition to its anti-inflammatory, anti-apoptotic properties. However, these versatile agents have never been investigated in microvascular regeneration processes.

In our study, in addition to haematological and haemorheological studies, we also analysed biomechanical and histopathological characteristics to assess the effect of PACAP and/or haemostatic sponge, BGP-15 or even inadequate tissue wetting on the regeneration of microvascular anastomoses in rats.

2 AIMS

1. Development of an in vivo model to study the regenerative processes of microvascular anastomoses
2. Objective assessment of the mechanical integrity (tensile strength) of vascular sutures, further development of the device
3. Follow-up study of the effect of pituitary adenylate cyclase activating polypeptide (PACAP) on the "maturation" of anastomoses
4. Investigation of the effect of BGP-15 on anastomosis healing
5. Collect qualitative and quantitative data on the significance of intraoperative inadequate tissue wetting

3 MATERIALS AND METHODS

3.1 Experimental permit

Our experiments were conducted in accordance with the regulations of the National Food Chain Safety Office (Act XXVIII of 1998 on the protection and welfare of animals) and the European Union regulations on animal experiments (EU Directive 63/2010). Our research plan was registered and approved by the Animal Welfare Committee of the University of Debrecen (permit registration number: 21/2022/DEMÁB).

3.2 Experimental animals and drugs used

Male Wistar (Crl:WI) rats (Toxi-Coop Zrt., Budapest, Hungary) were used in our studies. The animals arrived at the Department's animal facility at six weeks of age, where they acclimatised for 2-3 weeks. During preparation, the animals were weighed and then anaesthetised under general anaesthesia with a combination of ketamine and xylazine (100 mg/kg; 10 mg/kg). To prevent thrombosis, we administered heparin (HEPARIBENE, Teva Gyógyszergyár Zrt., Debrecen) through the lateral tail vein (80 NE/kg). For postoperative pain relief, we excluded traditional NSAIDs so that we could evaluate the healing processes without other influences, therefore we administered Tramadol (15 mg/body weight kg/day) intraperitoneally at the end of the surgery and on the first three postoperative days.

3.3 Surgical protocol

The surgical areas were depilated and disinfected (Betadine, Egis Gyógyszergyár Zrt., Budapest). An incision approximately 4 cm long was made on the inner thigh of the right hind limb, below and parallel to the inguinal ligament. We used an ALPHA ENT-4A (Elektro Optika, Érd, Hungary) microscope for microsurgical procedures. The fatty connective tissue surrounding the artery was dissected from the inguinal ligament to the origin of the saphenous artery and popliteal artery, separating it from the femoral vein and nerves. The artery, vein and inferior epigastric nerve (in PACAP-treated animals, these structures were transected), as well as the femoral vein and nerve and their lateral branches, were treated in a function-preserving, atraumatic manner and, if necessary, pulled aside without damaging the structures. One clip of the approximator was placed next to the ligament and the other in front of the distal branch of the femoral artery. The vessel was cut perpendicularly between the two with scissors. After heparin irrigation (~0.4 mL, 2500 IU/mL), we rinsed with physiological saline and used gauze to absorb the excess. After minimal adventitia removal, we performed eight simple knotted sutures with polyamide-6 monofilament suture material (Daclon, SMI, Vith, Belgium) containing 10/0 serrated needles. To ensure proper alignment of the vessel edges, the anterior and posterior walls of the vessel were separated using two opposing holding sutures. The

holding sutures were cut so that one thread was left longer (~ 3 mm) while the other was cut directly next to the knot. Using the remaining longer thread, we carefully manipulated the vessel edges so that they fit together properly, facilitating the formation of the appropriate stitch depth and distance. We then applied a new central support stitch at the midpoint of the support stitches, followed by additional midpoint stitches between the central and support stitches.

Once the front wall was complete, we cut the suture material next to the knot at each stitch (except for the outer retaining stitches). By turning the approximator and positioning the two outer retaining stitches correctly, the back wall was exposed, into which we placed three more stitches in a similar manner. Finally, we cut the longer threads of the two outer retaining sutures next to the knot. This simple but carefully designed knotting technique allows for precise alignment of the vessel ends and easy manipulation during surgery. After removal of the approximator, only mild leakage was observed in most cases. The patency of the anastomosis was checked in all cases using the 'milking test'. In the case of BGP and dissection vessels, the flow was also examined quantitatively (T206, Transonic Systems Inc., USA).

At the end of the surgery, the wound edges were wiped with Betadine gauze and the skin was closed with a horizontal mattress suture. During the follow-up period, wound checks were performed daily and Betadine wound care was performed as needed.

3.4 Sampling protocol

For laboratory measurements, we cannulated the lateral tail vein (26 G) on the preoperative day and on days 7, 14, and 21 after surgery, through which we collected 0.5 ml of blood (K3-EDTA, Vacutainer®, Becton Dickinson GmbH, Franklin Lakes, NJ, USA), followed by 0.8 ml of physiological saline solution. At the end of the surgery, after removing the cannula, we administered an additional 1 ml of physiological saline solution subcutaneously for fluid replacement.

At the end of the three-week follow-up period, the specified tissue samples (anastomosis and intact contralateral femoral artery; in the case of desiccation research, the inferior epigastric artery and vein as well) were removed and the animals were euthanised. The femoral arteries were dissected from the internal iliac branch to the popliteal bifurcation. After removal, the vessels were subjected to tensile strength testing as soon as possible. The animals were given an intravenous solution of ketamine-xylazine (50 mg/kg body weight; 5 mg/kg body weight) after both vessels had been removed so that the concentrated solution would not influence the test results.

3.5 Experimental Groups and Supplementary Procedures

3.5.1 *Investigation of the effect of PACAP 1-38 on vascular healing*

Thirty-four male Wistar rats (321.23 ± 37.1 g) were randomly assigned to four groups according to the treatments: SHAM (n = 8), BP (bioplast, hemostatic sponge) (n = 8), PACAP (n = 8), and PACAP + BP (n = 8). The fate of the histological samples taken during the study is shown in Figure 11. PACAP groups received 0.2 µg PACAP 1-38 (100 µg/vial, provided by Professor Gábor Tóth, University of Szeged) every other day, starting immediately after surgery. The compound was dissolved in 0.2 mL of physiological saline and flushed with an additional 0.2 mL saline through the cannula. The other groups received 0.4 mL of physiological saline via the same route at the same intervals.

In the BP groups, a 3 × 4 mm piece of Spongostan Standard™ hemostatic sponge (Raritan, Franklin Township, NJ, USA) was placed around the anastomosis site. In the PACAP + BP group, the first dose of PACAP was directly injected onto the sponge at the end of surgery, followed by wound closure using 4/0 polyglycolic acid (Surgicryl Rapid, SMI, Vith, Belgium).

Several options were considered for the pharmacological treatment method. Intravenous administration via the tail vein was ruled out due to the systemic load and the short half-life of PACAP. Local injection was also dismissed due to lack of standardization and high variability. From an animal welfare perspective, both methods would have required repeated anesthesia or significant stress every other day.

Considering these factors, we designed custom-made cannulas for local delivery. These were precisely positioned near the target vessel in the subcutaneous space, with an external access point located between the scapulae, out of the animals' reach, allowing for stress-free administration. A blunt 22G 1× needle (Neoject, Dispomed GmbH&Co. KG, Gelnhausen, Germany) was inserted into a 20 cm polyethylene tube (0.965 mm outer diameter, approx. 0.05 mL volume; Polyethylene Tubing, Clay Adams, 427411, BD Intramedic™, Sollentuna, Sweden). Each cannula was adapted to the size of the animal, taking into account their typical curled resting posture.

For fixation, 2 mm holes were drilled in both wings of the needle using a drill bit. Cannulas were disinfected inside and out with Betadine, then flushed with physiological saline prior to implantation. A subcutaneous tunnel was created from the lumbar region using a Lumniczer dissector, leading to the interscapular region where a small incision was made, and the prepared cannula was inserted. The wound was closed above and below the needle with Donati sutures (4/0 polyglycolic acid, Surgicryl Rapid), and the needle was secured with two simple loop knots (3/0 silk, braided, SMI). The distal end of the cannula was positioned

approximately 1 cm above the vessel and fixed in three points using surrounding tissues (8/0 polyamide-6, monofilament, Vitrex, Herlev, Denmark). A small hole (1.05 ± 0.034 mm) was cut directly above the anastomosis for local drug delivery.

3.5.1.1 Microcirculatory Measurements

Microcirculatory measurements were performed pre-operatively, 3 minutes after arterial clamping, at the end of surgery, and on days 7, 14, and 21 post-operatively. Measurements were taken on the lower part of the distal footpad on the lateral side of the hind paw. We used an LD-01 Laser Doppler flowmeter (Experimetria Rt., Budapest, Hungary) with a standard "pencil probe" (MNP100XP, Oxford Optronix Ltd., Adderbury, UK). The device provides a relative parameter of microcirculatory perfusion (blood flow units, BFU), influenced by the speed and number of moving red blood cells but not by their direction.

Each measurement involved a 20-second BFU recording, which was averaged. Skin temperature was monitored at the measurement sites using a ri-thermo® N professional clinical thermometer (Riester, Phoenix, USA).

3.5.1.2 Tissue Culture

In a preliminary study, we finalized the culture procedure for vascular samples. Portions of excised vessels were cultured in HG-DMEM medium (Dulbecco's Modified Eagle Medium, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Gibco, Gaithersburg, MD, USA) and antimicrobial agents (penicillin, streptomycin, ampicillin, fungizone + ascorbic acid). Vessel fragments were placed in Petri dishes (Eppendorf, Hamburg, Germany) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 14 days.

Some samples were treated with PACAP every 48 hours (10 µL, 10 nmol/L), according to the medium change schedule of each group. These vessel samples were derived from animals previously belonging to SHAM or treatment groups in vivo. In addition to intact and anastomosed arteries, a control group with freshly prepared, mechanically undamaged arteries was used as a reference. From two rats, four femoral arteries were dissected and halved using microsurgical scissors. These vessel segments (~4 mm) were similar in size to the anastomosed ones. Both proximal and distal segments were used equally in the in vitro experiments, and the same treatments were applied as in the in vivo experiments, except for the reference group, which had no surgical intervention or treatment.

3.5.1.3 Immunohistochemistry

Localization of collagen type I and elastin was visualized in vessel samples. Samples were fixed in 4% formaldehyde and washed in 70% ethanol. After embedding, serial sections were

made, followed by paraffin removal and rinsing in PBS (pH 7.4). Non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA; Amresco LLC, Solon, OH, USA).

Sections were then incubated overnight at 4 °C with monoclonal anti-collagen type I (1:500 dilution; Sigma-Aldrich, St. Louis, MO, USA) and polyclonal anti-elastin (1:1500 dilution; Sigma-Aldrich). Alexa Fluor 555-conjugated anti-rabbit secondary antibody (1:1000; Life Technologies Corporation, Carlsbad, CA, USA) was used for visualization. Nuclei were stained with DAPI using Vectashield mounting medium (Vector Laboratories, Peterborough, UK).

Negative controls were processed using only the Alexa Fluor 555 secondary antibody, without the primary antibody. Light microscopy images were acquired using a DP74 camera on an Olympus Bx53 microscope (Olympus Corporation, Tokyo, Japan). The Cell Sense Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan) was used for image acquisition with constant camera settings to allow for comparison of fluorescence intensities. Alexa555 and DAPI channels were merged using Adobe Photoshop 10.0. Contrast was uniformly enhanced without altering other settings.

3.5.1.4 Western blot

Coin samples were washed in physiological saline and stored at -70 °C. Samples were mechanically homogenised in liquid nitrogen using a tissue grinder. Then, 100 µL of homogenizing RIPA buffer (Radio Immuno Precipitation Assay; 150 mM sodium chloride, 1.0% NP40, 0.5% sodium deoxycholic acid, 50 mM Tris, pH 8.0) containing protease inhibitors (Aprotinin - 10 µg/mL, 5 mM benzamidine, Leupeptin - 10 µg/mL, trypsin inhibitor - 10 µg/mL, 1 mM PMSF, 5 mM EDTA, 1 mM EGTA, 8 mM sodium fluoride, 1 mM sodium orthovanadate). Suspensions were sonicated by pulsed ultrasound at 40 A for 30 s (Cole-Parmer, Vernon Hills, IL, USA). Whole cell lysates were prepared for Western blot analysis. 20 µg of protein was separated on 7.5% SDS-polyacrylamide gel for the detection of type I collagen, elastin and actin. The proteins were electrophoretically transferred onto nitrocellulose membranes and incubated overnight with the primary antibodies at 4 °C at the dilution indicated above. After washing in PBST for 30 min, the membranes were incubated with peroxidase-coupled secondary antibody: anti-rabbit IgG (1:1500; Bio-Rad Laboratories, Hercules, CA, USA) or anti-mouse IgG (1:1500; Bio-Rad Laboratories, Hercules, CA, USA). For signal detection, an enhanced chemiluminescence method manufactured by Advansta Inc. (Menlo Park, CA, USA) was used according to the manufacturer's instructions. Actin (housekeeping protein) was used as an internal control, and the expression results of elastin and type I collagen were normalized to it. Signal visualization was performed using a gel documentation system

(Fluorchem E, ProteinSimple, San Jose, CA, USA). The optical density of the signals was measured using ImageJ 1.40 g software.Examination of the effect of BGP-15 on vascular healing

Thirty-two male Wistar rats (330.2g±17.5g) were divided into four equal groups according to treatments: SHAM, cannulated SHAM (KSHAM), BGP-15 control (KBGP) and BGP operated (BGPO) groups. In the KSHAM and BGPO groups, a cannula was implanted, which was consistent with the protocol of PACAP treated animals. Animals in the pharmacon-treated group received 15 mg BGP-15 every other day until day 14. The KSHAM group was treated with the same amount of physiological saline. Femoral artery anastomosis was not performed only in the KBGB group, where the animals were treated subcutaneously without surgery to observe the effect of BGP. At the end of the interventions, the inguinal wound was closed with a 4/0 polyglycolide-poly (e-caprolactone) copolymer (Simfra, KOLLSUT, USA) suture with a reverse cutting needle.

3.5.2 *Research on the importance of keeping fabrics moist*

A total of 8 male Wistar rats (body weight: 307.54 ± 27.69 g) per group, 24 in total, were used to prepare vascular anastomoses. The animals were divided into Unfollowed Control (UNK), Operated, Desiccated groups. Anastomosis was performed in all three groups, however, in the UNK group no follow-up was performed, anastomoses were examined fresh after suturing. In our Operated group, special care was taken to keep the surgical site moist during the operation (31±3.5 min), this was done with physiological saline (Isotonic saline 0.9%; B. Braun Avitum Hungary; Hungary), while in our other non-wetted (desiccated) group we took extra care not to moisten the area by the surgeon, but also to separate the vessel from interstitial fluid and any fluid accumulating in the wound area. For wound closure, we used 4/0, reverse cutting needle, polyglycolide-poly (e-caprolactone) copolymer (Simfra, KOLLSUT, USA) suture material.

3.6 Laboratory Examinations

3.6.1 *Hematological Parameters*

Hematological measurements were performed using a Sysmex K-4500 automated hematology analyzer (TOA Medical Electronics Co., Ltd., Kobe, Japan). In our studies, red blood cell count (RBC, $x10^{12}$/L), white blood cell count (WBC, $x10^{9}$/L), and platelet count (PLT, $x10^{9}$/L) were determined based on the aperture impedance principle, while hemoglobin concentration (Hgb, g/L) was measured using a spectrophotometric method. From these data, the analyzer calculates the mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin

(MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), and hematocrit (Hct, %).

3.6.2 Micro-Rheological Parameters

3.6.2.1 Measurement of Red Blood Cell Deformability

Red blood cell deformability is characterized by the elongation index (EI), which can be plotted against the corresponding shear stress values (SS, Pa) on deformability curves. In our experiment, we used the LoRRca MaxSis Osmoscan ektacytometer (RR Mechatronics International BV, Zwaag, The Netherlands), which operates based on the principle of laser diffraction ectacytometry. The device emits a laser beam that scatters at the interface of suspending media with different densities, and the resulting diffraction pattern corresponds to the degree of cell elongation, i.e., their deformation.

The sample is placed in the gap (0.3 mm) between two concentric cylinders, and shear stress is generated through rotational motion. For the measurements, 10 µL of blood anticoagulated with K_3-EDTA is suspended in a high-viscosity polymer solution (~1 mL; 360 kDa polyvinylpyrrolidone, PVP; viscosity: 28 mPas; osmolarity: 294 mOsm/kg; pH = 7.3–7.4) immediately before testing. The high-viscosity medium facilitates proper alignment of the cells and effective transmission of shear stress to their membranes, which leads to elongation. Without this, the force transfer would be ineffective, and cells would merely rotate at different speeds, resulting in erroneous measurements.

The computer determines the rotational speed of the cylinder (and thus the shear stress) and analyzes the resulting diffraction pattern accordingly. All measurements were performed at a controlled standard temperature of 37 °C to simulate in vivo conditions.

Based on the diffraction pattern, the elongation index (EI) can be calculated using the major (A) and minor (B) axes of the elliptical pattern: $\mathrm{EI} = \frac{A-B}{A+B}$. The higher the EI, the more elongated the cells are. At a given shear stress, a higher EI indicates better red blood cell deformability.

EI and the corresponding shear stress data pairs were analyzed using a Lineweaver–Burke-type non-linear regression analysis, from which the maximum elongation index at infinite shear stress (EI_max) and the shear stress corresponding to half of this value ($SS_{1/2}$, Pa) can be determined: $\frac{1}{1/\mathrm{EI}} = \frac{\mathrm{SS1/2}}{\mathrm{EImax}} * \frac{1}{SS} + \frac{1}{\mathrm{EImax}}$.

Comparisons between different elongation index–shear stress curves can be made either at specific shear stress values (e.g., EI at 3 or 5 Pa), or using the derived parameters describing

the curve (e.g., EI_max, $SS_{1/2}$). With impaired deformability, EI_max decreases, and $SS_{1/2}$ increases, meaning that their ratio also declines.

3.6.2.2 Measurement of red blood cell aggregation

The red blood cell aggregation capacity was determined using the Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Roetgen, Germany), which operates on the principle of light transmission. A blood sample is collected in a measuring chamber system consisting of a 2º ground-glass lens and a cone-plate glass slide. 20 µl of anticoagulated blood (K3-EDTA, 1.5-1.8 mg/ml) is dropped into the centre of the lens, where it is spread in a circular pattern by folding the slide over the lens cone. An infrared diode is positioned above the glass system, the intensity variation of which through the sample is recorded by a detector positioned below. The sample chamber is rotated by a motor at a fixed speed of 600 Hz. This causes the cells to disaggregate, resulting in a decrease in the light transmission of the sample. When the motor is stopped abruptly or reduced to a speed of 3 Hz, the cells begin to aggregate and the light intensity increases steadily. The M parameter indicates the complete stop of the motor when the shear stress suddenly drops to 0, while the M1 parameter shows the data measured at 3 Hz speed, i.e. at very low shear stress, similar to physiological conditions. Aggregation indices were determined from light intensities at 5 and 10 seconds from the disaggregated state. From these we derived the four dimensionless parameters we also measured (M 5s; M 10s; M1 5s; M1 10s), whose increases indicate increased aggregation handedness.

3.7 Tensile strength measurement

An improved, custom-made instrument was used to measure the tensile strength, which was developed in collaboration with the Department of Information Technology, Faculty of Informatics, University of Debrecen and the Department of Surgery, Faculty of General Medicine. An early version of the device has been further developed based on experience.

3.7.1 Building a stable framework

One of the most important aspects in the design of the device was the design of a stable and strong frame. During measurement, significant forces are applied to the structure, so it is essential that the instrument does not move or vibrate excessively in order to ensure accurate data acquisition. This represents a significant improvement on the previous version, which was made from a simple metal box and allowed greater torsion, leading to less accurate measurements. In addition to a robust design, a lightweight yet durable construction that could be easily transported between laboratory, operating theatre and educational sites was an important consideration. Therefore, the frame was constructed from high-strength aluminium profiles.

3.7.2 Precise design of the traction mechanism

Accurate mechanics are essential for reliable measurements. The pulling unit is based on a HPV7 C-Beam linear actuator (Guangzhou Hanpose 3D Technology Co., Ltd., China) connected to a C-shaped aluminium structural element. The moving mechanism is driven by a NEMA17 stepper motor (42BYGHW811M, Kuongshun Electronic Ltd., China) controlled by a DRV882 motor controller (Pololu Robotics and Electronics, USA) built on an Arduino CNC Shield V3 (Kuongshun Electronic Ltd., China). The motor is coupled via a clutch to a T8 trapezoidal spindle, which moves a stall-free trapezoidal nut, allowing the C-beam carriage to perform precise linear motion.

The previous device used a plastic belt to pull the clamps, resulting in a non-linear transmission and variable speed. The winding belt increased the diameter of the shaft, causing each step of the motor to exert more force, and the belt's elasticity reduced efficiency and accuracy. In addition, the old pliers could only grip samples thinner than 2 mm.

During development, we first tried 3D printed grippers, but they were not stable enough under the high pulling forces. In the end, we used industrial standard grippers type HJJ-001 (Baoshishan Co., Dalian, China), which can withstand forces up to 500 N. The gripper surface is ribbed to prevent slipping or damaging the sample.

3.7.3 The force measuring system and its calibration

A tensile force cell with a tensile measuring stamp and IP 65 protection (TAL220, Deecom Technology, Kuala Lumpur, Malaysia) was used to measure the tensile force, complete with an analogue-to-digital converter. The strain gauge stamp converts mechanical changes in the fixed body into electrical resistance changes. As the body is deformed, the attached strain gauge stamp undergoes elastic deformation, while the resistance of the conductors inside it changes. The type we have chosen is a widely used load cell, often used in test equipment, measuring instruments, compression and tension devices. It contains two parallel mounted strain gauges with a measuring capacity of 5 kg (49.03 N). The device was used at constant room temperature under laboratory conditions to ensure comparability of measurements. As a result, the error and nonlinearity is below ±0.05%FS, virtually undetectable.

A high-precision 24-bit analog-to-digital converter HX711 (Avia Semiconductor Xiamen Ltd., Xiamen, China) was used to digitize the signal. The HX711 is specifically designed for strain gauges and other load cells. To ensure the accuracy of the load cell data, the instrument had to be calibrated. Calibration ensures that the measuring instrument is accurate and reliable. Calibration required the use of standard weights. Since the cell structure deforms during measurements, it is recommended that calibration be repeated annually. To eliminate

measurement errors, several odd number of measurements are taken in succession at the highest possible reading rate within a measurement period. The data are stored in buffer memory and the median value is transmitted to the computer. In this way, erroneous, extreme values are filtered out.

The most important difference from the first version was that the sensor had a wider measurement range, which allowed a wider variety of samples to be tested.

3.7.4 The starting and end positions

To limit the mechanical movement, limit switches (B073TYWX86, Qianxin Electronics Co., Ltd., Shanghai, China) were incorporated to prevent the trolley from exceeding the allowable range of motion, which could lead to irreversible damage to the gauge. In the case of the first version of the device, the measurement continued until the predetermined number of steps was reached, so that if the pulley system reached the end point before the end of the measurement sequence, the device would continue to pull, and could damage itself. Conversely, limit switches mounted on the pulling mechanism prevent the carriage from reaching the limits of its range of motion, such as hitting the clamp with the load cell.

3.7.5 Microcontroller

The Arduino Mega 2560 (RS-75-12, MEAN WELL Enterprises Co., Ltd., New Taipei, Taiwan) controller board handles the 24-bit signal from the A/D converter, controls the motor, monitors the switches while communicating with the computer in real time. The panel has 54 digital input/output connections (I/O ports), 16 of which can be used as PWM (pulse width modulation) outputs and another 16 as inputs for analog signals. The card has a USB, a power supply and an ICSP (In-Circuit Serial Programming) connector. Power for the complete device is provided by a 72 W power supply module (RS-75-12, MEAN WELL Enterprises Co., Ltd., New Taipei, Taiwan).

3.7.6 The software

The first version of the device was a desktop application written in C#, and the machine communicated with the computer via a USB 2.0 port. In the current version, we started using a universal serial monitor (QtSerialMonitor 1.5, open source software developed by Michal W. from GitHub Inc., San Francisco, California, USA). This allows us to issue commands to the device (e.g., moving the jaws to a predetermined position, adjusting the motor speed, starting the measurement), create backups (continuous log file saving), and export the measured data to a specific location in a CSV file.

3.7.7 Calibration

We encountered two problems when calibrating the device: the force sensor was designed to measure horizontal forces, and the distance between the two jaws was only 15 cm, so it was not possible to calibrate it with a simple standard weight. The solution was to rotate the entire device 90 degrees and place it in a horizontal position. We then made test weights of the appropriate size, placed them between the jaws, and secured them with the fastening strap at the top. The Arduino library (HX711_ADC) for the sensor also includes a calibration program (Calibration.ino 1.2.11, Olav K., open source software, GitHub Inc., San Francisco, California, USA), which helps to determine the calibration constant that can then be used in our own program. After the device determined the value 0 for the empty state, we reinforced the weights we had made and then weighed them with a certified laboratory scale (AJCS Laborscale, Demandy, Budapest, Hungary). We entered this value into the program, which then returned a constant value – this became the calibration number. The measurement was performed ten times with each weight (n = 5), the results were averaged, and the obtained value was entered into the Arduino program code.

3.7.8 Measurement implementation and evaluation

The removed arteries were always fixed in the clamping jaws at the same location, 8 mm apart, and the anastomosis site was centred. The pulling force exerted by the motor (for PACAP-treated vessels: 48.66 steps/min; 1.95 mm/s; in other studies: 4.78 steps/s; 0.1912 mm/s) was recorded in grams and exported to a CSV file, which was analysed using Microsoft Excel 2016. During data processing, we performed the gram-Newton conversion (9.81 m/s^2) and analysed the maximum value (maximum tensile strength) and the slope of the force-elongation curve at different points.

The analysis was performed on the curve section above 0.0098 N, as there were no irregular elements in this range based on the accuracy of the instrument. Since the fracture pattern of the curves was similar to that of elastomers, we ignored the initially more irregular first third and analysed only the slope of the ascending section (tgα; between 33–100% of the total rupture curve), which provides information on collagen integrity and elasticity, the component primarily responsible for the mechanical stability of various tissues.

3.8 Histology

Histological examinations were performed at the Institute of Anatomy and Developmental Biology. The samples were washed three times in PBS, then fixed in 4% formalin, and finally embedded in paraffin. After serial sectioning, hematoxylin-eosin (H&E, Sigma-Aldrich, St. Louis, MO, USA) staining was performed for morphological analysis, and orcein staining

(Sigma-Aldrich, St. Louis, MO, USA) was performed to visualise elastin. The staining protocols were performed according to the manufacturer's instructions. Microscopic were taken using an Olympus Bx53 microscope and a DP74 camera (Olympus Corporation, Tokyo, Japan). Non-specific picrosirius staining (Sigma-Aldrich, St. Louis, MO, USA) was used to examine the orientation of collagen fibres in blood vessels. By rotating the plane of polarised light by lambda/4 (Olympus Bx53 polarisation microscope), the structures showing double refraction indicate collagen fibres, where the bright red structures are thicker, while the green structures show thinner collagen fibres.

To measure the thickness of the tunica intima, media and adventitia, we examined 20× magnified photomicroscopic images of H&E-stained samples using ImageJ 1.40 g software. A perpendicular line was drawn from the membrana elastica interna towards the tunica adventitia, and the pixel values were determined.

The results were given as a percentage of the control pixel values. Twenty independent measurements were performed per section, and several independent arteries were used per experimental group. For orcein staining, we inverted the normal colour (green and black) to increase pixel contrast. The number of green pixels was determined using ImageJ software. We examined an equal area of the vessel wall at 40× objective magnification to determine orcein (green) positivity.

3.9 Statistical analysis

The planned sample size (individuals) was determined using the G*Power 3 program based on the Mead equation.

GraphPad Prism 9.1.2 (v.226) software was used for statistical analysis. We checked the normality of all data distributions and used Student's t-test or non-parametric tests (Wilcoxon or Mann-Whitney test) and two-way/repeated measures ANOVA tests accordingly. The significance level was set at $p < 0.05$.

4 RESULTS

4.1 Investigation of the effect of PACAP 1-38 on wound healing

4.1.1 General observations

All operations were performed successfully and in a standard manner. The weight of the animals in all groups decreased significantly after surgical trauma until day 7, then returned to baseline values by day 21.

Respiratory parameters were similar in all groups during anaesthesia; they increased significantly only at the end of the operation, when the effect of the anaesthetic had already worn off (BP: $p = 0.0216$; PACAP: $p = 0.0424$).

At the end of the follow-up period, moderate aneurysm formation was observed at the anastomosis site in all groups (SHAM: 2; BP: 3; PACAP: 1; PACAP + BP: 2). Arterial occlusion was observed in four animals (SHAM: 2; BP: 2).

4.1.2 Microcirculation

The plantar skin temperatures measured immediately before the microcirculation measurements were almost identical for both legs, with the highest values measured after surgery (SHAM left: 31.08 ± 2.63 °C, right: 30.47 ± 2.97 °C; BP left: 31.73 ± 0.98 °C, right: 31.74 ± 1.36 °C; PACAP left: 33.28 ± 0.96 °C, right: 33.09 ± 1.1 °C; PACAP + BP left: 32.01 ± 1.68 °C, right: 31.916 ± 1.11 °C). With the exception of the SHAM group, we observed a decrease in the other groups as the days progressed, with the lowest values measured on day 21 (SHAM left: 30.93 ± 0.81 °C, right: 30.95 ± 0.72 °C; BP left: 29.22 ± 1.38 °C, right: 29.84 ± 1.88 °C; PACAP left: 30.19 ± 1.27 °C, right: 30.09 ± 1.21 °C; PACAP + BP left: 29.2 ± 1.19 °C, right: 29.48 ± 0.97 °C).

The circulatory parameters (BFU) of the right and left feet changed in almost the same way during the interventions. During ischaemia, circulation parameters decreased in all groups except for the operated foot in the PACAP + BP group. Postoperative values were almost identical to those during ischaemia. The lowest postoperative values were as follows: SHAM: day 21, BP: day 14, PACAP: day 14 (left leg $p = 0.0323$), PACAP + BP: day 21. During the postoperative days, circulation gradually increased, reaching maximum values on day 14 in the PACAP + BP (right leg $p = 0.0291$) and SHAM groups (left leg $p = 0.0392$; right leg $p = 0.0004$) on day 14. The highest values were measured on day 7 in the BP group and on day 21 in the PACAP group (right leg $p = 0.002$).

4.1.3 Haematological changes

With the exception of the PACAP and BP groups, platelet counts increased significantly after surgery (SHAM day 14: $p = 0.025$; PACAP + BP day 7: $p < 0.0001$) and then normalised in almost all groups by day 21. The red blood cell count decreased until day 14, after which it began to normalise. The white blood cell count increased in all groups on day 7 after surgery (SHAM: $p = 0.0267$; BP: $p < 0.0326$; PACAP: $p = 0.0242$; PACAP + BP: $p < 0.0003$), most notably in SHAM animals, and then began to decrease. A slight time lag was observed in the PACAP-treated groups, with the maximum reached on day 14, followed by a decrease, but on day 21 there was still a significant difference ($p = 0.0026$) compared to the pre-surgery status. Haemoglobin and MCV parameters decreased continuously after surgery until day 21, but there was no significant difference between the groups. Haematocrit also decreased until day 14 and then increased by day 21.

4.1.4 Red blood cell deformability and aggregation

Red blood cell deformability showed a slight deterioration on day 7 (PACAP group EImax: $p = 0.023$ compared to baseline) and day 14 (PACAP + BP group EImax: $p = 0.0012$ and SS1/2: $p = 0.0039$ compared to baseline), but normalised in all groups by day 21.

Red blood cell aggregation deteriorated postoperatively in all groups. This was observed on day 7 p.o. in the M 5s and M1 5s parameters, especially in the treated groups compared to baseline values (M 5s: SHAM $p = 0.0278$; BP $p = 0.0027$; PACAP $p = 0.0012$; PACAP + BP $p < 0.0001$; M1 5s: BP $p = 0.0008$; PACAP $p = 0.0296$). On day 21, only the PACAP group showed a significant difference (M 5s: $p < 0.0001$; M1 5s: $p = 0.0163$). An increase was observed in the M 10s and M1 10s parameters on day 7, most notably in the BP group (M 10s: BP $p < 0.0001$; PACAP $p = 0.0078$; M1 10s: BP $p = 0.005$ compared to baseline). This deterioration persisted on day 21 only in the BP group (M 10s: BP $p = 0.0014$ compared to baseline).

4.1.5 Measurement of tensile strength

In all cases, the anastomosed femoral artery was significantly weaker than the intact contralateral artery. Compared to the contralateral artery, the lowest values were found in the BP group, and the strongest anastomoses were found in the PACAP group (SHAM: 0.647 ± 0.073 vs. 0.198 ± 0.089 N; BP: 0.641 ± 0.088 vs. 0.163 ± 0.059 N; PACAP: 0.63 ± 0.15 vs. 0.395 ± 0.177 N; PACAP + BP: 0.694 ± 0.061 vs. 0.325 ± 0.173 N). The tensile strength of the anastomoses was significantly ($p <0.0001$) reduced compared to native arteries (100%): SHAM anastomosis: 30 ± 10.4% ($p <0.0001$ vs PACAP), BP: 25.9 ± 12.3% ($p < 0.0001$ vs. PACAP; $p = 0.0456$ vs. PACAP + BP), PACAP: 57.8 ± 8.3% ($p = 0.0137$ vs. PACAP + BP), PACAP + BP: 39.6 ± 7.5%. The slope of the ascending section of the rupture curves decreased compared to the intact artery. In the 33–100% section of the curves, the BP group showed the greatest deviation compared to the intact artery (28.3 ± 8.9%), while the smallest deviation was in the PACAP group (64.8 ± 22.5%). The other two groups showed nearly identical differences (SHAM: 53.5 ± 13.4%; PACAP + BP: 55.8 ± 16.8%). The slope of the anastomosed vessels was compared to their own ipsilateral arteries (100%), and all showed significant differences ($p <0.0001$): SHAM group: 50.3 ± 10.2% ($p = 0.0021$ vs BP), BP: 24.5 ± 9%, PACAP: 53.7 ± 13% ($p = 0.0015$ vs. BP), PACAP + BP: 52.6 ± 10.2% ($p = 0.0039$ vs. BP).

4.1.6 Histology, molecular biology

4.1.6.1 *Thickness of vascular layers*

The thickness of the arterial walls decreased after BP treatment ($p = 0.0058$). Combined PACAP + BP treatment had a protective effect against this decrease. In anastomosed arteries,

the tunica intima did not change after BP or PACAP treatment, but a slight, non-significant increase was observed compared to SHAM anastomoses. The thickness of the tunica media increased significantly in PACAP + BP-treated samples ($p = 0.0121$) compared to other anastomosed arteries. The decrease in tunica adventitia wall thickness was significant in BP-treated anastomoses compared to SHAM, while PACAP treatment resulted in a slight decrease. Combined treatment reduced the decrease in tunica adventitia thickness in anastomosed vessels.

The walls of intact vessels (intact contralateral vessels as reference) did not show significant changes regardless of PACAP treatment, and even in vitro PACAP treatment following traumatic disruption of the vessels did not cause significant changes in anastomosed vessels. In contrast, PACAP increased the wall thickness of arteries. Separate analysis of the tunica intima showed no significant changes in either intact or anastomosed vessels following in vitro PACAP treatment. The thickness of the tunica media decreased slightly in in vitro cultured vessels, but there was no significant difference in either group. Interestingly, PACAP treatment decreased the thickness of the tunica adventitia in in vitro cultured vessels, but caused a significant increase in anastomosed vessels.

4.1.6.2 *Elastin expression*

The amount of elastic fibres in the vessels was visualised and quantified using orcein staining. In the anastomosed arteries, the intensity of orcein staining decreased in the BP group compared to the intact and SHAM anastomosed vessels. In contrast, the addition of PACAP to BP increased the intensity of orcein staining in anastomosed vessels in vivo. We also determined the number of elastin lamellae in the tunica media and observed some differences between the intact arteries of the different groups. The presence of BP caused a slight decrease in the number of elastic rings in the anastomosed tunica media. PACAP treatment increased the number of elastic lamellae in these vessels, but the application of BP reduced the effect of PACAP in the anastomosed arteries. Orcein positivity was also measured in the connective tissue surrounding the cannula, where PACAP could diffuse. Although elastin positivity was low in the vicinity of the vessels, a slight but not significant increase was observed after PACAP administration.

In vitro cultured vessels, without intervention, showed no significant change in orcein positivity after PACAP administration, but their orcein positivity was significantly higher than in operated vessels. In operated vessels, PACAP caused a slight but not significant decrease. The presence of PACAP did not cause any change in the anastomosed vessels. Similar to in vivo experiments, the number of elastic lamellae increased slightly with the addition of PACAP in in vitro tissue cultures.

Elastin protein expression was also examined by Western blot analysis. Intact and anastomosed vessels showed low elastin expression in the SHAM groups. PACAP and BP treatment increased elastin expression in both intact and anastomosed arteries. Furthermore, the addition of PACAP with or without BP increased elastin expression in anastomosed vessels compared to intact arteries. A similar increase in expression was observed in PACAP-treated in vitro tissue cultures.

Elastin immunopositivity was also demonstrated by immunohistochemistry. Diffuse signals were observed in the intact vessels of the SHAM and BP groups, with some lamellae showing immunopositivity. Elastin-positive lamellae were observed in the PACAP-treated groups. In PACAP-treated anastomosed vessels, diffuse and lamellar elastin-positive signals became more pronounced compared to intact vessels. BP treatment reduced the signal of elastin-positive lamellae in anastomosed vessels. Similar results were observed in cultured vessels, where strong diffuse elastin signals were visualised in intact in vitro cultures. PACAP treatment increased lamella organisation compared to anastomosed in vitro arteries.

4.1.6.3 *Type I collagen expression*

Type I collagen in the tunica media of arteries influences vascular integrity, so we examined the presence of collagen using picrosirius staining.

In intact vessels, BP reduced the amount of thick collagen fibres but increased the amount of thin collagen fibres in the tunica media. PACAP mainly caused a decrease in thin fibres, but increased the amount of thick fibres in anastomosed vessels. In in vitro tissue cultures, we examined anastomosed and then ruptured (traumatised) vessels. In this case, PACAP treatment slightly increased the presence of thick collagen fibres without changing the thin fibres. A similar amount of thick collagen fibres to intact arteries was observed in the SHAM groups, but a decrease was observed in PACAP-treated vessels. The number of thin fibres decreased in both SHAM and PACAP tissue cultures. The number of thick fibres decreased in anastomosed vessels compared to intact and anastomosed SHAM groups. The number of thick and thin collagen fibres decreased significantly in PACAP-treated in vitro anastomosed cultures.

We further examined the specific expression of type I collagen by Western blot analysis and obtained similar results in vivo. PACAP administration reduced expression in anastomosed vessels, but the presence of BP did not cause any change. In vitro tissue cultures showed little expression of type I collagen, but a slight increase was observed in anastomosed cultures. The localisation of type I collagen was also monitored by immunohistochemistry.

The tunica media of SHAM and treated arteries showed diffuse and fibrous immunopositivity. In intact arteries, PACAP and BP application reduced diffuse type I collagen signals and similarly reduced collagen fibres, as shown by picrosirius staining results. Type I collagen immunopositivity in anastomosed vessels was further reduced, and fibre signals disappeared in the PACAP-treated and BP groups. In tissue cultures, diffuse and fibre signals were observed in intact vessels, where PACAP administration further increased the collagen fibre content of the tunica media. In anastomosed vessels, type I collagen fibres were observed in the tunica media. PACAP administration increased diffuse collagen signs but did not enhance fibre formation.

4.1.6.4 *Granulomatous tissue around the cannula*

Pericannular granulomatous tissue formed in all groups, but it was more pronounced in PACAP-treated cases. In picrosirius-stained sections examined under polarised light, no significant increase was observed in the PACAP-treated groups in either red (SHAM and BP: 8030.26 ± 1224.92; PACAP and PACAP + BP: 17,954.93 ± 5,270.92) and green (SHAM and BP: 3,575.05 ± 2,248; PACAP and PACAP + BP: 9,203.76 ± 3,876.22) light densities. There was no visible difference in the orcein-stained sections in the PACAP-treated groups (SHAM and BP: 1212.869 ± 183.353; PACAP and PACAP + BP: 1382.403 ± 93.97) in terms of integrated light density.

4.2 Examination of the effect of BGP-15 on vascular healing

4.2.1 General observations

No aneurysms or thrombosis were encountered during the study. The flow parameters hardly changed during the surgery, only in the KSHAM group a significant change was observed compared to baseline (p=0.0116) and SHAM (p=0.0203). On day 21, comparing anastomotic flow with the data from the own intact contralateral arteries, a significant increase was observed in the BGPO group, both on the intact and anastomosed side. These data were measured at almost the same time (anaesthetic depth) per animal, so when looking at the relative data, there was no difference between the groups (SHAM: 95.70%, KSHAM: 1.18%, BGP: 0.99%)

The animals tolerated well the general care procedures, the bi-daily treatments and the wound cleansing. This was mainly observed in BGP treated animals. In general, they were more relaxed compared to their peers. The weight of the animals started to increase after the surgical trauma and, with the exception of the BGPO group, exceeded the baseline value by day 21, with significant increases in the SHAM (p=0.0132) and BGPK (p=0.0303) groups.

4.2.2 Laboratory tests

4.2.2.1 Haematological measurements

Although BGP-15 was delivered locally, directly to the site of anastomosis, it did not only affect blood vessels, but could also be absorbed into the circulation and thus exert systemic effects. In the KSHAM group, significant persistent leukocytosis developed and persisted from day 7 to day 21, indicating a prolonged inflammatory response induced by the surgical trauma and the cannula. In contrast, only a lower, transient increase in FVS was observed in the BGPK and BGPO groups, with a decrease below baseline in the BGPK group on day 14, confirming the anti-inflammatory effect of BGP-15.

In the BGPO group, both red blood cell count and hematocrit values showed a significant and continuous decrease, especially on days 14 and 21 (VVS: 7.04±0.46 and 7.06±0.34; Htc: 38.96±2.7 and 39.41±2.5). In the BGPK group, however, a milder decrease was observed, which was close to normal by day 21. In contrast, in the other groups, there was only a significant difference from baseline values in the first week after surgical trauma. Haemoglobin levels in the BGPO group were also significantly lower than in the other groups on days 14 and 21. This was paralleled by a decrease in red blood cell count and haematocrit, whereas the BGPK group showed only a slight but consistent decrease by day 14. In the SHAM and KSHAM groups, the more significant difference in haemoglobin levels was limited to day 7.

In the BGPO group, significantly lower MCV values were measured at all time points, which may indicate a shift towards microcytosis. The BGPK group also showed a smaller but statistically significant decrease in the early postoperative weeks. In the control groups, MCV showed no clinically relevant change. BGPO animals developed persistent thrombocytosis with significantly higher values from day 7 onwards. No significant changes in platelet counts were observed in the BGPK group and KSHAM group. In the SHAM group, a transient postoperative increase in platelet count was observed, which decreased from day 14.

4.2.2.2 Red blood cell deformability and aggregation

EImax values showed small variability in all groups, but the differences were not clinically significant. For SS1/2, a significant decrease was observed in the KSHAM group at day 7 ($p=0.0071$ vs baseline), which may indicate a deterioration in VVS deformability. No such difference was observed in the BGPO and SHAM groups. In contrast, the BGPK group without surgical stress had an elevated SS1/2 value at day 7 ($p=0.0023$ vs baseline value), which may indicate a deterioration of deformability.

For red blood cell aggregation, with respect to the M 5s parameter, a significant increase in aggregation was observed in the groups receiving BGP (BGPK and BGPO) from day 7 onwards

(*p<0.05 vs SHAM, KSHAM), which persisted to day 21. A marked increase was also observed for the KSHAM group, but this was nearly normalized by day 21. A similar trend was observed with longer aggregation time (M 10s parameter). In the SHAM group, a postoperative transient increase was observed, while in the BGPO group a significant significant increase was observed by day 21 (p=0.001). For M1 5s and M1 10s (aggregation at low shear stress), an increase in M1 5s was measured in the BGPK group by day 21, indicating an increase in postoperative aggregation. In the BGPO group, we also saw higher values, especially for the M1 10s index, indicating a sustained increased tendency to aggregation.

4.2.3 Tensile strength measurement

Intact arteries had significantly higher tensile strength than anastomosed ones. BGP treatment increased the tensile strength of the anastomoses, but an increase was also observed in the KSHAM group. The steepness of the curves in the range 33-100% (collagen dominated irreversible deformation stage) showed the greatest deviation in the KSHAM group, while the BGPO group showed the least deviation compared to intact vessels, suggesting biomechanical maturation of the artery.

4.2.4 Histological studies

H&E stained sections were used to investigate the thickness of the vascular wall layers. For the total layer thickness, we observed a significant difference in the BGPO group compared to the SHAM group, which amplified the physiological intimal hyperplasia present in the SHAM group after wounding.

We examined the amount of type I collagen fibres in picrosyrus-stained sections. Non-significant but lower levels of thick (red) and thin (green) fibers were observed in anastomoses compared to intact vessels. The amount of thinner fibres in anastomoses of the BGPO group was slightly increased compared to the SHAM group.

The amount of elastic fibers and the number of lamellae they formed were examined in orcein stained sections. For the BGPO group, a significant increase in the amount of elastic fibres was observed, without any difference in the number of lamellae.

4.3 Research on the importance of tissue moisture retention

4.3.1 Macroscopic observations

During surgical procedures, we have observed that manipulation of non-moist tissue has become increasingly difficult. In the Dissected group, on day 21, there was increased dense connective tissue at the surgical site and thrombosis in one artery and aneurysm in another.

The epigastric vessels were not affected by incision and suturing injury, but the tissue area containing them was the most exposed to dehydration. The weight of the animals varied

similarly during the follow-up period, but the dehydration group had a much higher variance and weight gain was also lower compared to the Operated group.

All anastomoses were successfully performed in both groups, as shown by the pre- and post-operative arterial flow rates. At day 21, no significant difference was found between the two groups or between the two sides, but slightly increased blood flow was observed in the Dissected group.

The skin temperature of the animals did not change significantly, but the Desiccated group showed a slightly elevated plantar skin temperature on day 21.

4.3.2 Laboratory parameters

Slight differences were observed in haematological parameters. During the follow-up period, the white blood cell count did not change significantly. The red blood cell count showed a significant increase in both groups in the second week (Operated p=0.0169; Desiccated p=0.0018), and in the third week only the Operated showed a difference (p=0.0418). The hematocrit showed a significant increase in the Desiccated group compared to the Operated group in week 2 (p=0.0030). The only significant difference in haemoglobin concentration was in the Operated group at week 1 (p=0.0220). Platelet counts showed a slight increase in both groups, with the greatest increase in the Operated group, where it was significant (p=0.0007) at week 1. The MCV increased in the second (p=0.0212) and third (p=0.0233) week in the Desliced group. MCH increased in the Desiccated group in the first week (p=0.0168), whereas it decreased in the Operated group in the second (p=0.0002) and third (p=0.0007) weeks. MCHC also showed a significant decrease in the Operated group in the second (p=0.0003) and third (p=0.0001) weeks.

We also examined the deformability parameter of red blood cells. The curves of the Desequenced group showed the greatest decrease in the first week compared to baseline, indicating a deterioration in deformability. Interestingly, the curves of the Operated group showed an increase after the intervention. There was no significant difference between EImax and SS1/2 between the groups. The EImax/SS1/2 showed a significant decrease in the Desiccated group in the first week compared to Baseline (p=0.0014) and Operated (p=0.0471). EI 3Pa showed a decrease in the groups after surgery, but an increase in the third week, mostly in the Operated group (p=0.0061 vs. baseline).

The degree of red blood cell aggregation is expressed by the aggregation index. M 5s and M1 5s index parameters increased in the desiccated group after surgery and were significant in the first week compared to SHAM (M 5s p=0.0190; M1 5s p=0.0026), then normalized by the third week. The M 10s parameter showed an increase in the Operated group and a decrease

in the Desiccated group after surgery. This caused a significant difference between the two groups in the first week (p=0.0423), but normalized by the third week. The M1 10s parameter showed only small, non-significant changes.

4.3.3 Measuring tensile strength

Force-elongation diagrams were obtained from the tensile strength measurements and analysed numerically. The stress-strain curves of the arteries are similar to those of elastomers. The analysis determined the maximum tensile strength for which the anastomosis of all three groups was found to be significantly weaker compared to the intact contralateral artery (UNK p=0.0146; Operated p<0.0001; Dissected p=0.0006). There was no significant difference between the groups, but the strongest were the freshly made UNK anastomoses and the weakest were the Operated arteries.

The slope of the curves was examined for the entire section (0.001N- maximum point) and separately for the areas 0-33% and 33-100% of the entire section. For the full curve, the UNK anastomosis had the largest deviation compared to the native contralateral artery (p=0.0024), both in the initial part of the curve (0-33%) (p=0.0138 vs native base) and in the terminal part of the curve (33-100%) (p=0. 0138 vs native base). For the Dissected and Operated groups, the slopes were not significantly reduced in the full and terminal part of the curve compared to the intact contralateral artery. However, for the Desiccated group, an increase was observed at the initial stage compared to the intact contralateral vessel, which was found to be a significant (p=0.0175) difference compared to the UNK group.

4.3.4 Histomorphology

There was no significant difference between anastomoses in terms of total wall thickness and tunica adventitia thickness. However, intima thickness did not increase in the Dissected group. The tunica media thickness increased mainly in the Desiccated group (p=0.0127 vs. UNK).

In addition to the arterial wall layer thickness, a general morphological analysis was performed. The tunica intima was identified without pathological abnormalities in both the Operated and Desected groups. More swollen cells appeared in the tunica media of the Desiccated group and the total number of cells was increased compared to the Operated group. The tunica adventitia contained a slightly denser collagen matrix and slightly denser tissue, and it is likely that the number of fibroblasts was also increased in the Dissected group. On the other hand, the intensity of staining was higher in the tunica adventitia in the Desiccated group. This stronger eosinophilic appearance may indicate increased protein expression.

In the epigastric artery, only the tunica intima showed a significant increase in both the Operated (p=0.0073) and the Dissected group (p=0.073) compared to the intact vessel. In contrast, for the epigastric vein, a non-significant thickening of the tunica media, adventitia and total thickness was observed in the Desiccated group.

On picrosirius stained sections, both red (thick fibers) and green (thin fibers) light intensities showed an increase in the Desiccated group compared to intact arteries, and a significant (p=0.0226) increase in red light intensity was observed compared to UNK anastomoses.

We examined the amount and lamellar arrangement of elastic fibres in sections stained with orcein. For femoral arteries, we did not see clear differences in the amount of elastic fibers and lamellar arrangement. However, in the epigastric artery, we observed a non-significant increase in the Operated group, which was not observed in the Desected group. A small decrease in the volume of lamellae was observed in the Dissected group.

5 MAIN FINDINGS AND CONCLUSIONS

1. 1. We have successfully developed a model to investigate the regeneration processes of microvascular vascular anastomoses and to deliver different local treatments in a cost-effective and appropriately modulated manner.
2. 2. Objective investigation of the mechanical integrity (tensile strength) of vascular sutures has been further developed using a tensile strength measuring device that can be used for multiple tissue sample types.
3. 3 We reported for the first time the effects of PACAP and BGP-15 on microvascular anastomoses, taking into account systemic effects. PACAP application increased elastin expression, whereas BP decreased it, but no significant changes in type I collagen expression were observed. Biomechanical elasticity and tensile strength increased in the PACAP group, whereas they decreased in the BP group. However, their combined application was beneficial for vascular regeneration.
4. 4. Topical application of BGP-15 had a beneficial effect on the regeneration of microvascular anastomoses, improved the regulation of the inflammatory response, supported the process of biomechanical maturation and also promoted remodelling of the vascular wall at the structural level. However, the haematological and aggregation

changes observed as a consequence of systemic uptake, especially in the BGP-treated anastomosed group, require further investigation.

5. The objective data presented on the importance of inadequate intraoperative tissue wetting support the classical surgical principle. Further research is needed to optimise the frequency and duration of hydration in order to define the reversible/irreversible limit of tissue dehydration.

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List of publications related to the dissertation

1. **Fazekas, L.**, Handari, D., Tran, L. D. L., Varga, Á., Mátrai, Á. A., Fillér, C., Al-Smadi, M. W., Al-Khafaji, M. Q., Tóth, A., Juhász, T., Németh, N.: An old-new problem: the impact of intraoperative tissue desiccation on the regeneration of vascular anastomoses.
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List of other publications

4. Kincses, G., **Fazekas, L.**, Varga, Á., Mátrai, Á. A., Nguyen, X. L., Barabási, K., Flaskó, A., Juhász, T., Molnár, Á., Németh, N.: Following-Up Micro-Rheological and Microcirculatory Alterations During the Early Wound Healing Phase of Local and Rotated Musculocutaneous Flaps in Rats.
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