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Foreword

This is a rare book. Not because it will be difficult to find, but because its subject is seldom treated – and even neglected – in medical literature. And, this is not because microcirculation is difficult to research or because it is inaccessible, but because we have – in spite of abundant data – little understanding of how it is regulated, how it behaves in pathological conditions, and how we can control it therapeutically. Indeed, it is difficult to overestimate its importance. Life depends on an uninterrupted traffic of "supplies" to the individual cells, the evacuation of their waste products, as well as substances transmitting messages to and communicating with other distant organs or cells – microcirculation. I tend to think of microcirculation as a network of larger and smaller streets, passageways – at times, hardly passable footpaths – connecting larger avenues in a major metropolis. If these multitudes of intersections become jammed, collateral routes are taken. If this should also fail, the city's inhabitants would suffer, an entire district may be deprived of important supplies and communication, or the entire city could even die. This book expounds on the methods of exploration and our current understanding of that complex – but so essential – component of an evolutionarily advanced living organism with a fully developed circulatory system. Microcirculation signifies life. It assures that human beings can survive even up on Mount Everest, break the 100 meters' world record in swimming or sprinting. It signifies survival or – if insufficient – death, by sepsis, for example. Microcirculation signifies continuing to live or, if disturbed, stopping movement, breathing, thinking and even life itself.

Intravital microscopy, allowing the observation of microcirculation in living organisms, while organs and tissues are fully functioning, is one of the main methods of investigating microcirculation. It bridges the gap between the study of intracellular functions, on the one hand, and organ functions or clinical studies on the other. Indeed, the behavior of circulating individual cells can be observed. It is also possible to intervene in various ways, to influence the circulation and behavior of these circulating cells, or even to correct pathological behavior. In addition, with intravital microscopy, the effects of other techniques of molecular biology can also be observed in real life situations. Since it, more or less, uses classical microscopy techniques, there are inevitable limitations to

magnification and the possibility of penetrating deeper into the structures. Certainly, intravital microscopy is consistently overcoming limitations in comprehending intracellular functions, in visualizing intracellular processes and even in comprehending the molecular processes – unthinkable, just a short while ago. The new generation of intravital molecular imaging microscopy facilitates the direct visualization of not only normal physiological processes, but even specific molecular pathological processes. Pharmacological interventions allow observation of how active agents affect the behavior at cellular or even intracellular levels, permitting an explanation of pharmacological mechanisms in greater detail. The quantity of possible persistent artifacts in the course of in vitro experiments is reduced to a minimum during observation of the process in an intact organism. Intravital microscopy of microcirculation involves more than merely the observation of micro blood vessels. Alongside investigation of the purely circulatory processes, the responses of immune cells, coagulation processes, or the behavior and modulation of adhesion molecules could be studied using various experimental protocols.

This book is certainly not the first to describe microcirculation, as observed using the technique of intravital imaging of regions of the body, until recently inaccessible. It will be of interest to researchers or clinicians involved with circulation in the brain, liver, intestine, joints, sublingual area and the eye. This book is invaluable as a preparatory reference volume, elucidating various exploration techniques, or as a reference for clinicians, seeking to delve more deeply into the pathophysiological mechanisms involving microcirculatory disturbances. It is, in any case, a sincere invitation to this attractive, high technology research, which also offers the broad esthetic experience permitted by the visualization of living structures, a real spectacle of colors and the most fantastic life forms. Life's limitless inner beauty is a realm to discover.

Paris, November 1, 2012

Dragan Pavlovic
Research Director
Experimental Anesthesiology
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Vascular Access and Tracheostomy

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Purpose

Animal experiments for intravital microcirculation imaging may require vascular access for administration of fluorescence dyes. Depending on the experimental model intravascular catheters can be placed in the neck (jugular vein) or the groin (femoral vein). Arterial cannulation is used for invasive blood pressure measurement. Tracheostomy is feasible to secure the airway during the procedure.

2.1 Animals

After approval by the Institutional Animal Care Committee male Lewis rats (250 ± 50 g) are used in the experimental protocols described in this textbook. Animals are housed in chip-bedded cages and, prior to experiments, acclimatized for one week in the air-filtered institutional animal care facility of the Faculty of Medicine at Dalhousie University, Halifax, Nova Scotia, Canada. Animals are kept on a 12 hours light/dark cycle, with a constant room temperature of 22 °C and humidity of 55-60%. A standard diet of rodent chow and sterile drinking water are available *ad libitum*. All animals are housed in accordance with the standards and procedures set forth by the Canadian Council on Animal Care.

2.2 Materials

2.2.1 Chemicals and drugs

- Sodium pentobarbital (Ceva Sante Animale, Montreal, QC, Canada),
- Sodium chloride 0.9% (Hospira, Montreal, QC, Canada).

2.2.2 Surgical instruments

- Forceps – broad, fine tipped, and curved tipped (Fine Science Tools, BC, Canada and Codman, Massachusetts, USA)
- Golden A5 hair clippers (Oster, Tennessee, USA)

2.2.3 Disposables

- Intramedic Non-radiopaque polyethelene tubing (PE 50) (Clay Adams, Sparks, MD, USA)
- Intramedic Non-radiopaque polyethelene tubing (PE 100) (Clay Adams, Sparks, MD, USA)
- Silk black braided string (Ethicon, New Jersey, USA)
- Surgical tape (3M Transpore Medical, Minnesota, USA)
- General purpose non-woven sponges (Medicom, Quebec, Canada)
- Cotton tipped applicators (Puritan Medical Products Co, Maine, USA)
- Surgical mat
- Syringes (1 ml, 3 ml, 10 ml) (Becton Dickinson and Company, Franklin Lakes, NJ, USA)
- Needles (23 and 25 5/8 gauge) (Becton Dickinson and Company, Franklin Lakes, NJ, USA)
- Epoxy

2.2.4 Catheter assembly

Catheters are made by inserting a 23 gauge needle into Intramedic Non-radiopaque polyethelene tubing (PE 50) and secured by an epoxy adhesive. All necessary items for catheter assembly can be seen in Fig. 1. Forceps are used to place the PE 50 tubing on the 23 gauge needle to avoid injury. Then, PE 100 Intramedic Non-radiopaque polyethelene tubing is placed over PE 50 tubing where the needle and PE 50 tubing overlap. The larger tubing is secured to the PE50 tubing by epoxy to ensure leaks in the line do not occur due to a puncture in the PE 50 tubing.

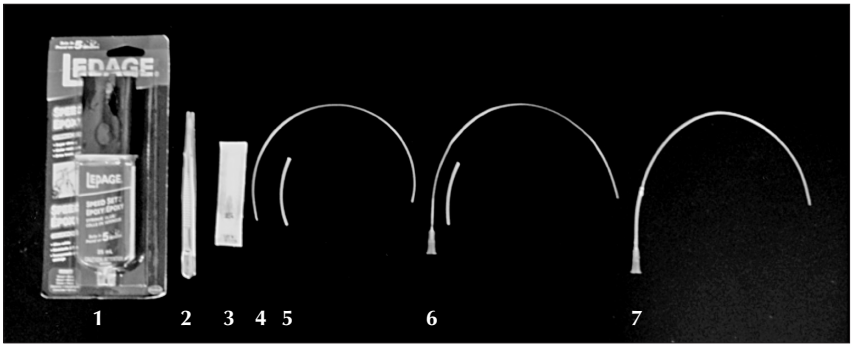

Fig. 1: *Catheter materials and assembly. 1) epoxy; 2) forceps for placing PE50 tubing onto 23 gauge needle; 3) 23 gauge needle; 4) PE 50 tubing; 5) PE 100 gauge tubing; 6) PE 50 tubing attached to the 23 gauge needle (epoxy applied); 7) PE 50 tubing attached to the 23 gauge needle with PE 100 tubing supporting the overlap of the needle and the PE 50 tubing to ensure no leaks.*

2.3 Preparation of the rat

After weighing (Fig. 2) the animals are anesthetized with 54.6 mg/kg (54.6 mg/ml stock solution) pentobarbital by intraperitoneal (i.p.) injection. Anaesthesia is maintained throughout the experiment by additional intravenous (i.v.)

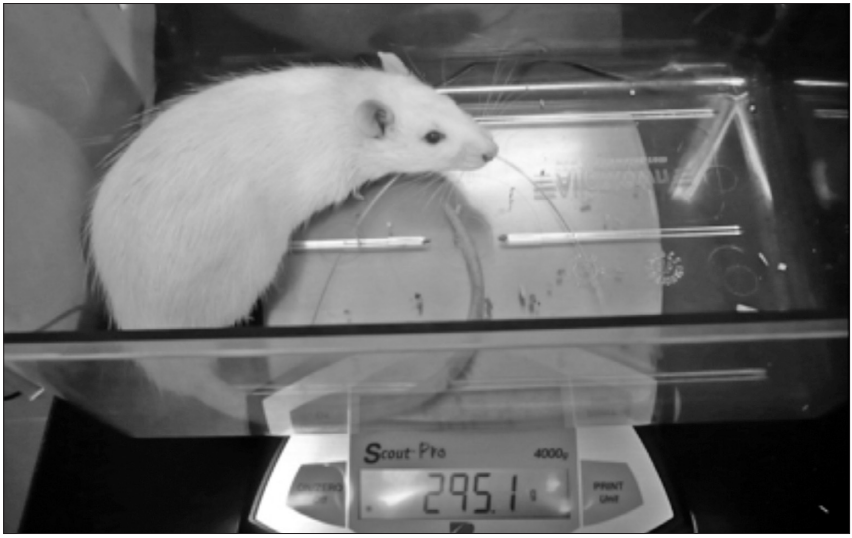

Fig. 2: *Weighing before anesthesia*

injection(s) of pentobarbital. Typically 0.05 ml of pentobarbital is given when the rat responds to a toe pinch. The animal is then prepared for surgery by removing hair from the lower abdomen and the ventral part of the neck. The animal is then placed in the supine position with limbs taped to a plexiglas plate for the duration of the surgery. Animals core body temperature is maintained at 37-38 °Celsius by the use of a heating pad.

2.4 Jugular vein and carotid artery cannulation

The equipment needed for the insertion of catheters can be seen in Fig. 3. Surgical instruments can be seen in Fig. 4.

The first step is a midline neck incision (Fig. 5). After incision, forceps and hemostats are used to dissect the (left) external jugular vein. Once identified, the vessel is completely dissected from the surrounding connective tissue (Fig. 6). Using silk string, a surgical knot is secured around the vessel distal to the forceps. A loose knot is then made proximal to the forceps (Fig. 7). Use micro-scissors to make a cut close to the distal end of the vessel. Using micro-forceps introduce the polyethylene catheter into the vessel (approximately 1 cm) as demonstrated in Fig. 8.

Fasten the catheter by tying surgical knots with the silk strings to attach the catheter to the vessel (Fig. 9). Next, dissect the (right) carotid artery and care-

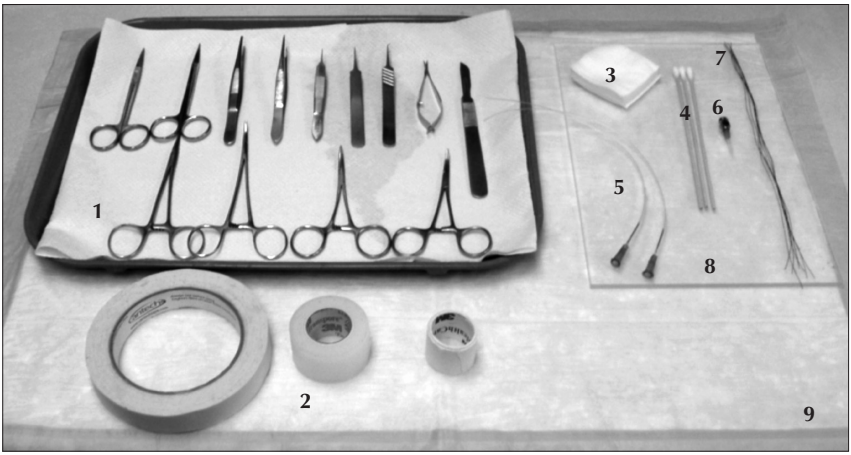

Fig. 3: *1 Surgical tools, 2 Tape, 3 Gauze sponges, 4 Cotton tipped swabs, 5 Cannulation lines, 6 16 gauge catheter for tracheostomy, 7 Silk string, 8 Plexiglass glass plate, 9 Surgical mat*

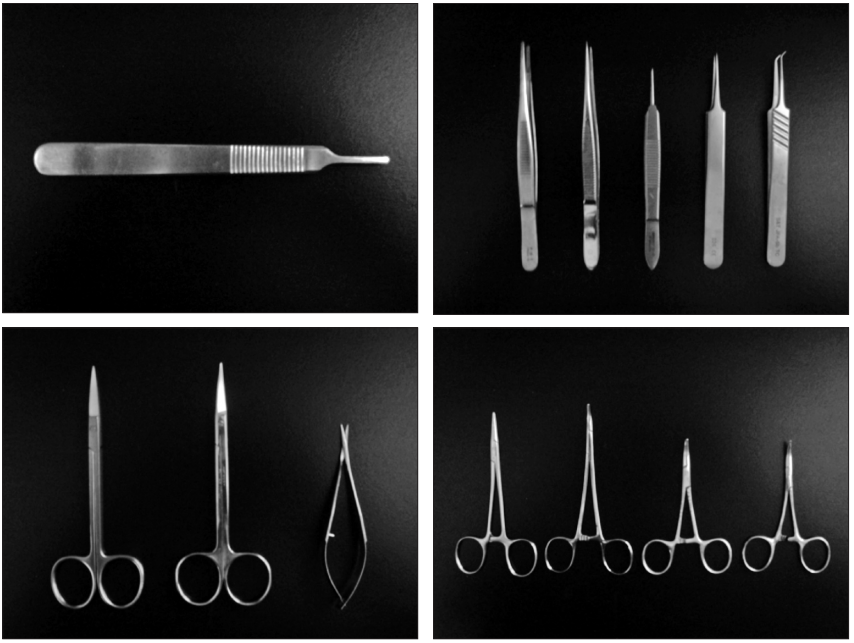

Fig. 4: *Surgical tools in order or right to left. Scalpel (no blade), forceps in order of tip diameter (wide to fine, then curved tip), scissors (straight, curved tip, micro-surgical), haemostats (6′ straight, 6′ curved fine tipped, 4′ curved blunt tipped, 4′ curved fine tipped)*

fully separate the vessel from the nerve attached to it as seen in Fig. 10. Perform the same procedure as for venous cannulation; however, a surgical vessel clamp should be used to hold the proximal end of the vessel as arterial blood

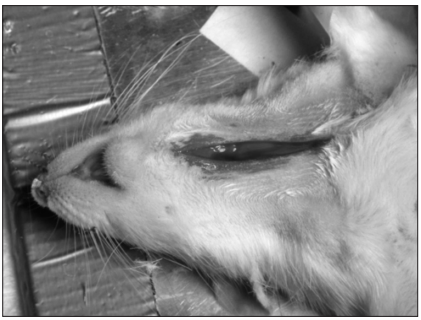

Fig. 5: *An incision was made in the midline of the neck*

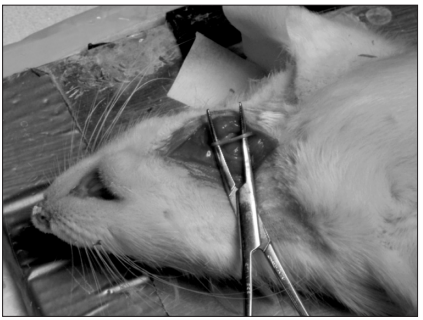

Fig. 6: *Forceps and hemostats were used to dissect the jugular vein.*

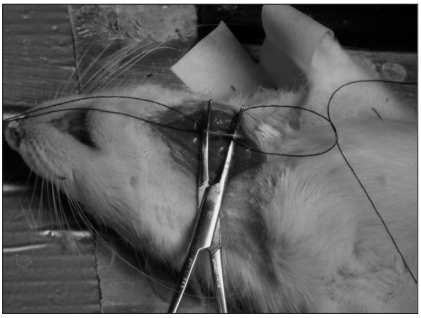

Fig. 7: *A surgical knot is tied at the distal end of the exposed vessel and a loose knot at the proximal side of the vessel using silk string.*

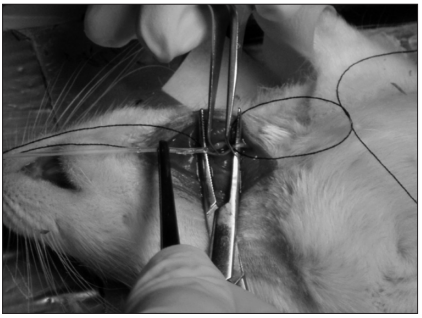

Fig. 8: *Micro-scissors are used to make a cut at the occluded distal end of the vessels and the polyethylene catheters are inserted to the vessel with forceps.*

pressure is high. It should be noted that the vessels should be kept moist with saline during the procedure as this will allow easier insertion of the catheter.

2.5 Femoral vessel cannulation

An incision is made in the inner thigh (approximately 2-3 cm) and the femoral artery and vein are cannulated with the PE 50 catheters. This is done by dissection of the femoral artery and vein by blunt forceps. Once the vein and artery are dissected the distal ends are tied off by sections of silk string. Tension is applied to the proximal end of the vessel by a loose knot and a hemostat as can be seen in Fig. 11. Each vessel is cut with micro-scissors at the distal end (Fig. 12). The curved fine tipped forceps are inserted into the vessel and used

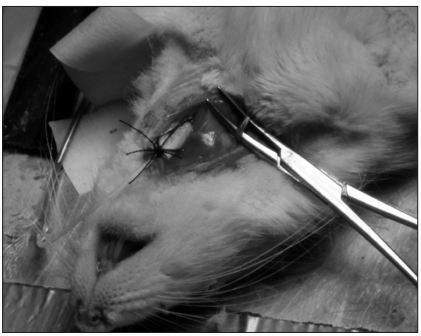

Fig. 9: *Dissection of the carotid artery from the nerve attached.*

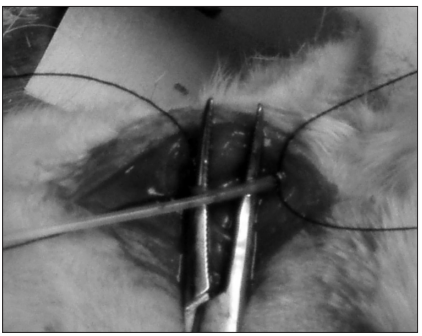

Fig. 10: *The catheter is secured by surgical knots.*

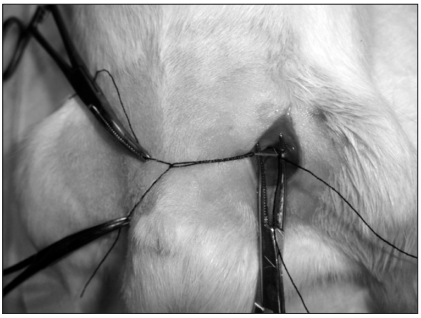

Fig. 11: *Femoral vessels dissected. Distal vessel end is occluded by surgical knots of silk string. The proximal end of the vessel is under tension by a loose surgical knot held in place by hemostats*

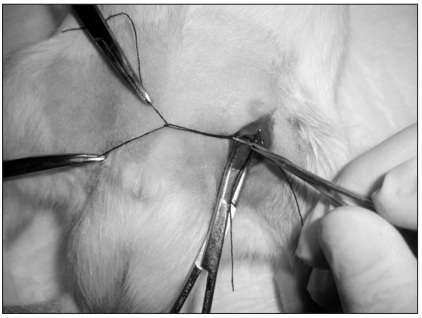

Fig. 12: *Microsurgical scissors are used to cut a small incision to the vessel while tension is applied to either end to stop bleeding.*

to open the interior so that the PE 50 catheter can be inserted into the vessel using forceps as seen in Fig. 13. Catheters are secured in place by firmly tying silk string on the proximal end of the vessel, which has the catheter in it. This is further secured by attachment of both proximal and distal string to a piece of surgical tape placed on the catheter.

2.6 Tracheostomy

A tracheostomy is conducted so that breathing could be assessed and the airway would not be obstructed due to mucus. Prior to this procedure silk string should be tied to the 16 gauge catheter which aids in securing the catheter to

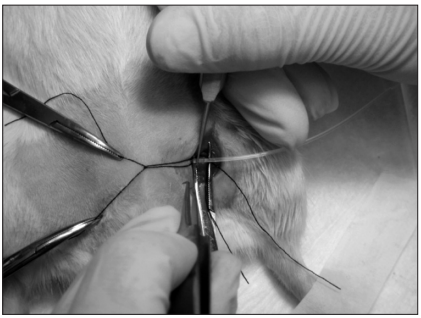

Fig. 13: *Fine tipped curved forceps inserted into the vessel incision to widen lumen, line inserted into the vessel. Proximal surgical knot to keep the catheter in the vessel.*

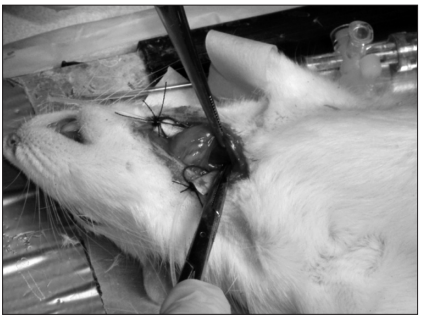

Fig. 14: *Dissection of the muscle tissue surrounding the trachea by use of forceps and haemostats.*

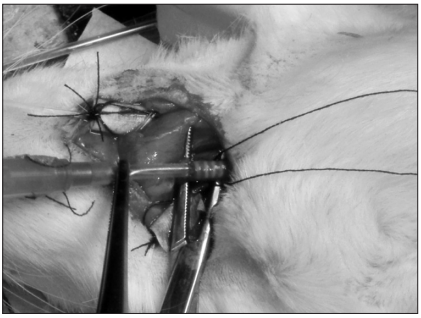

Fig. 15: *Tracheostomy being performed by insertion of a 16 gauge catheter being placed into an incision between the cartilage of the trachea*

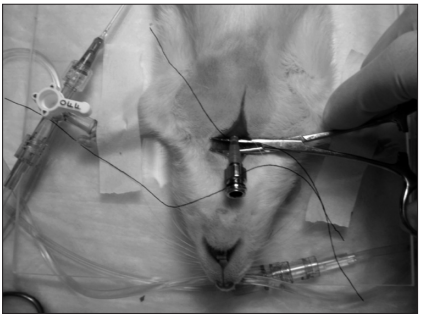

Fig. 16: *The catheter is firmly adhered to the trachea by tying silk string around the trachea catheter overlap.*

the trachea as described later. An incision will be performed on the ventral side of the neck, parallel to the trachea of the rat. It should be noted that this was already done if the jugular vein and carotid artery are cannulated. To perform the tracheostomy dissect the trachea from its surrounding tissues. The muscle tissue was dissected away from the trachea with blunt forceps (Fig. 14). Two silk strings were placed under the trachea so they could be tied to the trachea in the following steps. The trachea was lifted slightly and an incision was made between the annular hyaline cartilage in the trachea with the scalpel. Any blood in the surrounding area was removed. A 16 gauge catheter was placed into the trachea (Fig. 15). Insertion of the catheter was followed by securing (tie 2-3 surgical knots) the catheter to the trachea. The two sections of string around the trachea were firmly tied to provide an obstruction so fluid could not enter the trachea as seen in Fig. 16. A 3 ml syringe connected to a short cannula as suction was used for removal of fluids that might be obstructing the trachea.

2.7 Vital sign monitoring

The monitor for the measurement of arterial blood pressure (BP), heart rate (HR) and temperature (T) should be tarred before the experiment. Mean arterial pressure (MAP) and heart rate (HR) will be monitored and recorded every 15 minutes. A temperature probe is also attached to the monitor to record rectal temperature. Continuously body temperature will be maintained at 37 ± 0.5 °C by use of a heating pad which can be adjusted accordingly.

2.8 Troubleshooting

Insertion of a catheter into the vessels can often lead to problems, such as the vessel lumen being punctured by the catheter or trouble entering the vessel lumen. This problem can be avoided by cutting the PE 50 tubing at the end that is inserted into the vessel on a diagonal to the perpendicular of the tubing. Once this is done to create a tip, the point is rounded off by scissors so that the catheter does not puncture through the lumen of the vessel. During the insertion of the trachea catheter, blood from the surrounding vessels may occlude the tracheal pathway. Before the insertion of the catheter the area should be cleaned of blood as well as any from inside the trachea.

3

Intestinal microcirculation

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3.1 Type of research

- Measuring leukocyte-endothelial interactions in the submucosal collecting and postcapillary venules.
- Measuring functional capillary density in the longitudinal muscle, circular muscle and mucosal layer of the intestinal wall.
- Methods can be used for research, e.g. in sepsis, ischemia/reperfusion or inflammatory bowel disease.

3.2 Protocol summary

3.2.1 Surgical preparation (time required: 25-30 min)

- Placement and fixation of the rectal thermometer: 1 min
- Disinfection of the surgical area (neck): 1 min
- Incision and vessel dissection: 5 min
- Cannulation of the jugular vein and the carotid artery: 15 min
- Tracheostomy: 5 min

3.2.2 Preparation for intravital microscopy (time required: 10-15 min)

- Set-up of the stage (heating, superfusion): 2 min
- Disinfection of the area of the surgery (abdomen): 1 min
- Incision and preparation of the terminal ileum: 2 min
- Placement of the terminal ileum on the stage: 2 min
- Placement of the animal under the microscope: 2 min
- Administration of fluorescence dyes: 2 min

3.2.3 Microscopy (time required: 55-60 min)

- Leukocyte-endothelial interactions
  – recording of 5-10 collecting venules (each venule 30 sec): 10 min
  – recording of 5-10 postcapillary venules (each venule 30 sec): 10 min
- Capillary perfusion of the longitudinal and circular muscle layers
  – recording of 5-10 visual fields of the longitudinal muscle layer (each area 30 sec): 10 min
  – recording of 5-10 visual fields of the circular muscle layer (each area 30 sec): 10 min
- Mucosa preparation
  – Incision of the anti-mesenteric intestinal wall using microcautery: 2 min
  – Cleaning the intestine from feces, flushing with saline: 2 min
  – Placement of the coverslip: 1 min
- Mucosal capillary perfusion
  – recording of 5-10 visual fields of the mucosa (each area 30 sec): 10 min

3.3 Materials

3.3.1 Animals

- Male Lewis rats (body weight: 250 g ± 50 g)

3.3.2 Drugs and Chemicals

- Sodium pentobarbital (Ceva Sante Animale, Montreal, QC, Canada)
- Rhodamine 6G (Sigma-Aldrich, Oakville, ON, Canada)
- 5% Fluorescein isothiocyanate (FITC)-albumine, bovine (Sigma-Aldrich, Oakville, ON, Canada)
- Lipopolysaccharide from Escherichia coli, serotype O26:B6 (Sigma-Aldrich, Oakville, ON, Canada).
- Normal saline (0.9% Sodium Chloride, Montreal, QC, Canada)
- Potassium Chloride (EDM Chemicals Inc., Gibbstown, NJ, USA)

3.3.3 Equipment

- Monitor (Hewlett-Packard, Model 66S, Saronno, Italy)
- Epifluorescent microscope (Leica DMLM, Wetzlar, Germany)
- Light source (LEG EBQ 100, Jena, Germany)
- Lens (Leica N PLAN L 20X/0.40)

- Filter for use with Rhodamin 6G (Leica)
- Filter for use with FITC-albumin (Leica)
- Black and white DAGE CCD video camera (DAGE MTI Inc., Michigan City, IN, USA)
- Video tape recorder (DSR-25 DVCAM SONY, Halifax, Canada)
- Black and white monitor (Speco technologies, Texas, USA)
- Scalpel (Fine Surgical Tools, North Vancouver, BC, Canada)
- Surgical blade (size 10) (Lance Parafon Ltd., Sheffield, UK)
- Microcautery knife (Medtronic, USA)
- Gauze sponges
- Cotton tipped swabs
- Syringes
- G27 cannula
- Glass coverslip

3.3.4 Special equipment

- Intravital microscopy stage with hanging drop (Fig. 1)

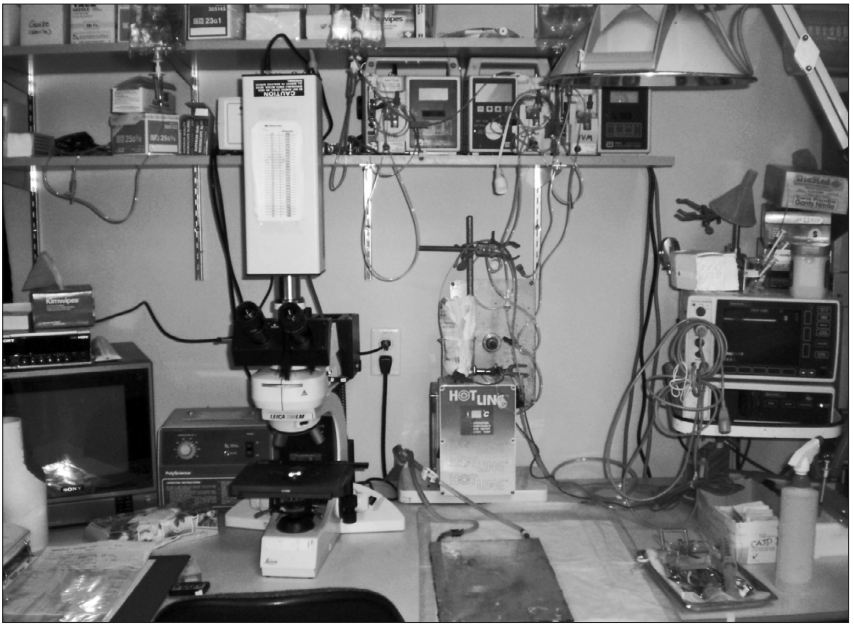


Fig. 1: *Intravital microscopy set-up*

3.4 Detailed description of procedures

3.4.1 Animal preparation

The described protocol was approved by the Animal Care Committee of the Carlton Animal Care Facility, Dalhousie University Halifax, NS, Canada, according to the Canadian Council on Animal Care guidelines.

3.4.1.1 Anesthesia

Rats were weighed with a commercially available scale. Afterwards an intraperitoneal administration of 60 mg/kg pentobarbital) was performed using a syringe with a G27 cannula, whereas great value was placed on blood free aspiration of peritoneal fluid. The injection site was located at hip level lateral to the linea alba, the animal's legs bent.

With the anesthesia deploying its maximum effect 15 to 20 minutes after administration the operative procedure began. Throughout the experiment sufficient depth of anesthesia was assessed by checking the animal's reaction to ear or tail pinch and, when needed, more anesthesia was administered in small intravenous dosages (up to 5 mg/kg body weight).

3.4.1.2 LPS and drug injection and monitoring

At time 0 min lipopolysaccharide (Escherichia coli, serotype O26:B6, Sigma-Aldrich, Oakville, ON, Canada) was administered intravenously. The process of LPS administration should be performed slowly (15 mins) and carefully, while monitoring the blood pressure (BP). The mean arterial blood pressure (MAP) should not be lower than 60 mmHg. If blood pressure or heart rate drops lower than normal range, oxygen should be administered via the tracheotomy until the animal recovers. Drug administration took place 15 minutes following LPS challenge. The drugs were administered via the jugular venous catheter. The cannabinoid drugs were previously dissolved in the vehicle dimethyl-sulfoxide (DMSO) (Sigma-Aldrich, ON, Canada) and administered over duration of minimum 15 minutes. During the two hours of observation period, mean arterial pressure (MAP), heart rate (HR) and temperature (T) was measured continuously. MAP and HR will be recorded every 15 minutes (Hewlett-Packard Monitor, Model 66S, Saronno, Italy). A temperature probe is also attached to the monitor to monitor rectal body temperature that was maintained at 37.5 °C by use of a heating pad (thermostatic platform), which can be adjusted appropriately.

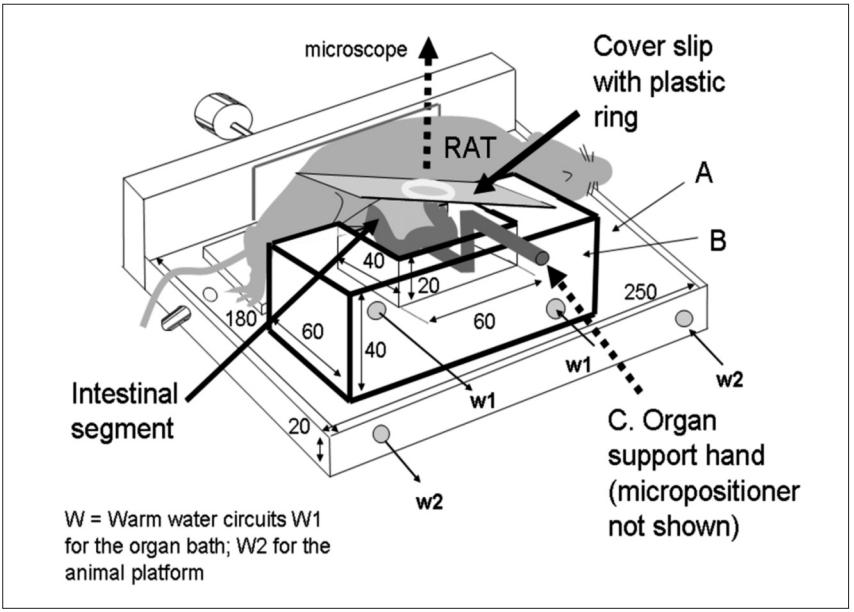


Fig. 2: *Hanging drop [5]*

3.4.1.3 Preparation for IVM

The next step was the preparation of the area of interest for IVM. Specifically a segment of the terminal ileum was chosen.

By adjusting the tube next to the intestine under the slide, the saline could pull itself between intestine and cover slip. Out of this tube heated saline with a temperature of 37 °C drips constantly. We use a flow of 5 ml/h to make sure, that the gut, which is swapped out of the abdominal cavity, is even warm and moist.

Use a cover slip (without any compression or traction of the gut) on section of the terminal small intestine that is chosen for the intestinal microcirculation (approximately one square cm). However, it is important to know that the intestinal wall can be brought close to the cover slip, or permitted to only touch it slowly [5].

IVM will be conducted using a 5-cm-long segment of the terminal ileum proximal to the ileocecal valve. The ileum segment will be supported using a cus-

tomized device with a cover slip. This arrangement allows approximately 1 cm^2 of intestinal tract to be accessible to microscopic investigation. Areas of the intestine not being subjected to examination will be covered with gauze and continuously superfused with saline kept at 37 °C to avoid dehydration and exposure to air.

3.4.1.4 Preparation of the microscopic area

First, turn on the Hotline system (Smiths Medical, Rockland MA), wait until the temperature reaches 37 °C. Next, arrange the life care 5000 infusion system (Absolute Medical Equipment, USA) at a continuous rate of 5 ml/h of saline (0.9% Sodium Chloride, Montreal, QC, Canada). At this point we can start the surgical procedure to reach the interest segment of intestine.

3.4.1.5 Laparotomy

Prior to this procedure, clean and disinfect the surgical area (abdomen), by using alcohol swabs. 30 mins prior to IVM (1.5 hour following onset of the experiment) laparotomy is performed and a 5 cm segment of the terminal ileum is exposed. To perform the laparotomy use a scalpel and make a superficial skin cut on the abdomen. Lift the muscular layer, use scalpel or curved tip scissors cut right along the linea alba to open up the muscular layer. The purpose of lifting the muscle layer is to avoid cutting and damaging the intestine and other organs in the abdominal cavity. After cutting the skin and the muscle layer, by use of cotton swabs soaked in saline exteriorize a 3-4 cm long segment of the intestine (terminal ileum) through the midline incision and place it carefully on the specially designed stage and hold it by a supporting device. Surround the portion of the intestine that is close to the animal with gauze sponges that are soaked in the saline (always keep the intestine moist). During the microscopic procedure, the intestine will be perfused with thermostat-controlled (37 °C/ 98 °F) saline solution to avoid drying. Place the animal while it is located on the heating pad, on the microscope stage.

3.4.1.6 Fluorescence staining

15 minutes before the start of the IVM leukocytes are stained in vivo by the i.v. injection of Rhodamine-6G (1.5 ml/kg) and the plasma is stained with FITC (1 ml/kg). FITC facilitates evaluation of the capillary flow by amplified contrast of the plasma. Rhodamine and FITC both were administered into the jugular vein and flushed with saline to ensure the complete administration. This process

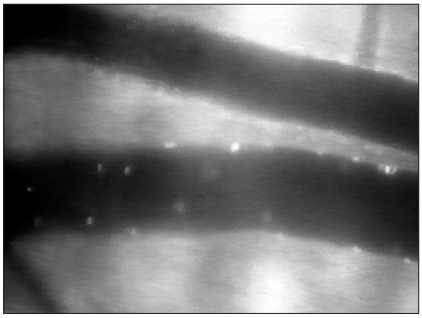


Fig. 3: *Grade I collecting venules (V1)*

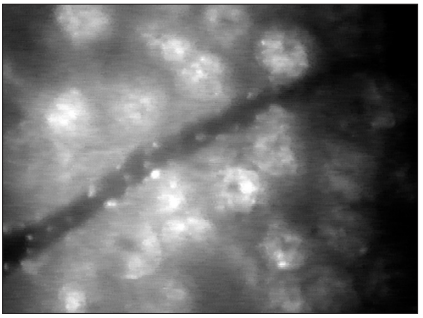


Fig. 4: *Grade III postcapillary venules (V3)*

occurred within the dark and fluorochromes are covered by aluminum foil to avoid light as they are photosensitive. Intravital fluorescence video microscopy will then be performed.

3.4.1.7 Leukocytes adherence

The microscope will then be set to focus upon the submucosa of the prepared intestinal section. Leukocytes will be visible in the venules. Six visual fields containing nonbranching grade I collecting venules (V1) over a length of at least 300 µm and six visual fields revealing similar grade III postcapillary venules (V3) will be observed and recorded for 30 seconds (Fig. 3, 4).

3.4.1.8 Functional capillary density

Next, change the filter for examinations with FITC-albumin, and focus setting. Video sequences (30 s) of six randomly selected fields of the capillaries within the longitudinal musculature will be recorded as well as six fields of the capillaries within the circular muscle (Fig. 5, 6).

3.4.1.9 Mucosa preparation

The examination of the mucosa is performed through an opening of the intestinal lumen over a length of 2 cm. Use microcautery knife (Medtronic, USA) to make a cut on the intestine, opposite to the direction of the mesenteric vessels. Choosing a section that is filled with faeces is preferred in order to avoid heat alterations of the intestinal mucosa. Followed by flushing with warm

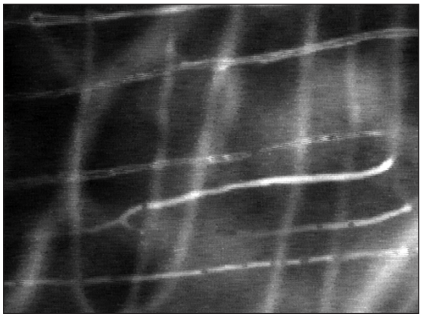


Fig. 5: *Longitudinal muscle*

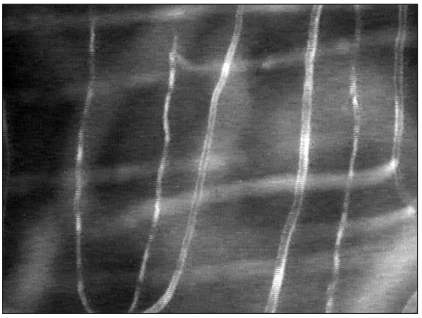


Fig. 6: *Circular muscle*

(37 °C) saline, the intestine will be lifted again and held by the supporting device. Next, use cotton swabs soaked in saline to remove the faeces. Place a cover slip on the prepared segment and perform the rest of IVM microscopy for the mucosa. Place the cover slip not too tight, and place the hanging drop tube again close to the mucosa. Six 30-s video sequences of randomly chosen mucosa sections will be recorded (Fig. 7).

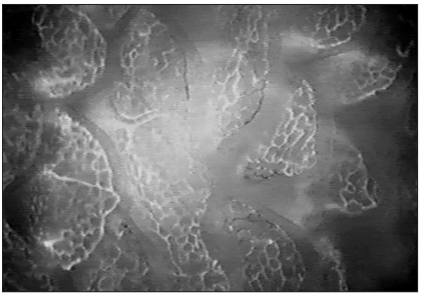


Fig. 7: *Mucosa of the small intestine*

3.4.1.10 Blood/tissue samples

At the end of all experiments, use a heparinized syringe to draw arterial blood samples (total volume, 1 ml). Follow by spinning at 5,000 rpm for 10 mins, then separate the plasma carefully. Store the plasma at –80 °C.

Tissue samples will be collected from the intestine. Half of the tissue samples will be fixed in formalin (10%) for histology and the remaining half will be frozen in liquid nitrogen for mRNA by RT-PCR and protein analysis by Western Blot (WB) to quantify receptors and signaling molecules.

The animals will be euthanized by Potassium Chloride (149 mg/ml saline, 149 mg/kg) (EDM Chemicals Inc. Gibbstown, NJ, USA).

3.4.1.11 Analysis

Evaluation of the video sequences will be carried out off-line on a video monitor. The following parameters will be analyzed: adhering leukocytes (the number of leukocytes that during an observation period stayed immobile for at least 30 s to an oblique, cylindrical endothelial surface); sticker (= cells per square millimeter), flow of rolling leukocytes (the number of leukocytes that during an observation period of 30 s pass in a rolling motion through a selected vascular diameter); roller-flow (= cells per minute). Vessel lengths and diameters were also recorded and measured. Functional capillary density (FCD) (the length of capillaries with observable erythrocyte perfusion in relation to an predetermined rectangular field); methodology according to Schmid-Schoenbein et al. (FCD = $cm/cm^2 = cm^{-1}$). According to his method we count the number of intersections between the capillaries and the grid, which is used to calculate the length of capillaries per area. This type of grid system contains *P* number of squares; the grid width, *d* that is defined by actual size of the grid divided by magnification factor. Using the point-counting method allows to measure the size of module by counting the number of points inside the module (P_M) by using the formula below:

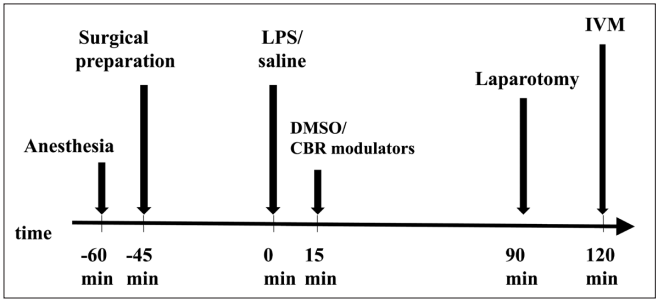
$$A_M = P_M \, d^2$$

A_M: the surface area of the module
P_M: number of squares with marked centerpoints
d: the grid width

$$L_C = \text{p number}/2. \; N_C/L$$

L_c: is the length of capillaries per unit area of the module
$L = 2P_M d$: is the total length of the grid system inside the module
N_c: is the number of interceptions between the grid and the capillaries

3.4.1.12 Example protocol



3.4.1.13 Example results

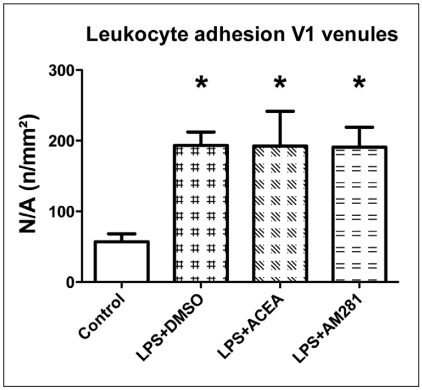


Fig. 8: *CB1R modulation does not affect intestinal leukocyte adherence in experimental endotoxemia. Leukocyte adhesion in collecting venules (V1) of the intestinal submucosal layer (N/A = number of adhering leukocytes per area; n = 10 per group); endotoxemic animals treated with placebo dimethylsulfoxide: LPS+DMSO; CB1R agonist: 2.5 mg/kg ACEA; CB1R antagonist: 2.5 mg/kg AM281. * p < 0.05 vs. Control animals.*

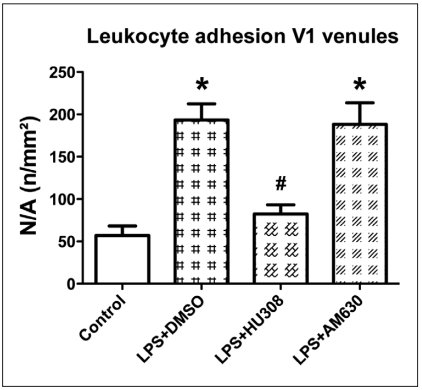


Fig. 9: *CB2R stimulation reduces intestinal leukocyte adherence in experimental endotoxemia. Leukocyte adhesion in collecting venules (V1) of the intestinal submucosal layer (N/A = number of adhering leukocytes per area; n = 10 per group); endotoxemic animals treated with placebo dimethylsulfoxide: LPS+DMSO; CB2R agonist: 2.5 mg/kg HU308; CB2R antagonist: 2.5 mg/kg AM630. * p < 0.05 vs. Control animals, # p < 0.05 vs. LPS + DMSO.*

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