Video Article

Fixed Volume or Fixed Pressure: A Murine Model of Hemorrhagic Shock

Lauryn K. Kohut[1], Sophie S. Darwiche[1], John M. Brumfield[1], Alicia M. Frank[1], Timothy R. Billiar[1]

[1]Department of Surgery, University of Pittsburgh

Correspondence to: Lauryn K. Kohut at taitlk@upmc.edu

Abstract

It is common knowledge that severe blood loss and traumatic injury can lead to a cascade of detrimental signaling events often resulting in mortality. [1, 2, 3, 4, 5] These signaling events can also lead to sepsis and/or multiple organ dysfunction (MOD). [6, 7, 8, 9] It is critical then to investigate the causes of suppressed immune function and detrimental signaling cascades in order to develop more effective ways to help patients who suffer from traumatic injuries. [10] This fixed pressure Hemorrhagic Shock (HS) procedure, although technically challenging, is an excellent resource for investigation of these pathophysiologic conditions. [11, 12, 13] Advances in the assessment of biological systems, i.e. *Systems Biology* have enabled the scientific community to further understand complex physiologic networks and cellular communication patterns. [14] Hemorrhagic Shock has proven to be a vital tool for unveiling these cellular communication patterns as they relate to immune function. [15, 16, 17, 18] This procedure can be mastered! This procedure can also be used as either a fixed volume or fixed pressure approach. We adapted this technique in the murine model to enhance research in innate and adaptive immune function. [19, 20, 21] Due to their small size HS in mice presents unique challenges. However due to the many available mouse strains, this species represents an unparalleled resource for the study of the biologic responses. The HS model is an important model for studying cellular communication patterns and the responses of systems such as hormonal and inflammatory mediator systems, and danger signals, i.e. DAMP and PAMP upregulation as it elicits distinct responses that differ from other forms of shock. [22, 23, 24, 25] The development of transgenic murine strains and the induction of biologic agents to inhibit specific signaling have presented valuable opportunities to further elucidate our understanding of the up and down regulation of signal transduction after severe blood loss, i.e. HS and trauma [26, 27, 28, 29, 30].

There are numerous resuscitation methods (R) in association with HS and trauma. [31, 32, 33, 34] A fixed volume resuscitation method of solely lactated ringer solution (LR), equal to three times the shed blood volume, is used in this model to study endogenous mechanisms such as remote organ injury and systemic inflammation. [35, 36, 38] This method of resuscitation is proven to be effective in evaluating the effects of HS and trauma [38, 39].

Video Link

The video component of this article can be found at http://www.jove.com/video/2068/

Protocol

1. Instrument and Surgical Field Preparation:

1. Instrument Preparation.

All surgical procedures are performed using aseptic techniques. A surgical blue pad and sterile field dressing are used. All materials and instruments are sterilized before use. 6-0 Suture, cotton tip applicators, gauze, male-male 3-way stopcocks, and instruments are autoclave sterilized. Transducers, PE-50, and PE-10 tubing are Ethylene oxide sterilized. All 3-way stopcocks, syringes and needles are received sterile.

6-0 Suture is cut into 1-inch pieces (6 pieces / animal) and put into small sterilization pouches. Cotton-tip applicators, 4x4 gauze squares, and male-male 3-way stopcocks are put into either small or medium sized sterilization pouches and autoclaved. Our surgical instruments are autoclave sterilized each evening. They are washed after surgery using antibacterial soap and tap water. They are allowed to dry on a clean surgical blue pad. They are then carefully placed into a sterilization pouch and sterilized for use the next day.

Since the transducers and tubing have plastic components, they must be sterilized using gas, i.e. Ethylene Oxide. PE-10 tubing is cut into 5-inch pieces and placed into a small sterilization pouch. PE-50 tubing is cut into 18-inch pieces and placed into a medium sterilization pouch.

2. Surgical Field.

To set-up the surgical field, first, turn on the hot bead sterilizer to ensure it reaches the appropriate temperature– 300-350°F before starting surgeries. Then proceed to the next steps by placing surgical blue pads down on an alcohol wiped benchtop. One pad goes under the microscope and the other goes on the circulating heating pad where the BP analyzers are located. Place a sterile field dressing over both

surgical blue pads. Fill a stainless steel instrument tray 1/3 of the way with 70% alcohol. Use enough 70% EtOH to cover all surgical instruments. Use a separate sterile field dressing and place it next to the microscope. Place all sterile instruments, suture, gauze, and catheters on this sterile field. Be careful when opening sterile instruments and suture Not to contaminate them by touching them. It is best to use sterile gloves when doing this setup procedure.

2. Mechanical Set-up and Procedures:

1. Catheter Set-up.

To set-up the right leg murine catheter used to measure BP, first, put on sterile gloves. Then get the sterile PE-10 tubing from the autoclaved pouch. Grab the middle of the tubing with pointer finger and thumb leaving about an inch between them. Stretch this section of the tubing just a bit to make it thinner in diameter to help with catheter insertion. After stretching the tubing cut it in half using sterile scissors. The 5-inch tubing should now be two pieces approximately 2 ½-inches in length. Make sure to bevel the stretched end.

*Do NOT angle the beveled edge too much as this could increase the chances of exiting the lumen by poking through the underside of the vessel wall.

Insert a 30G needle into the blunt unstretched end of the tubing. Get a sterile 1cc syringe and a 3-way stopcock. Use an alcohol wipe to sterilize the top of the 10cc sterile vial containing the heparinized saline solution (0.1ml Heparin/9.9ml Saline). Fill the syringe with 0.6-0.7cc of the heparin solution. Attach the 30G needle and catheter to the end of the 3-way that is directly across from the male end. Fill the stopcock, 30G needle, and PE-10 tubing with the heparin solution. Make sure to get ALL the AIR BUBBLES OUT of the system. The most effective way to remove all the air bubbles is to use gravity. Point the needle toward the ground while letting the tubing dangle to the benchtop. Give the needle hub a flick of the fingers and the bubbles will float to the top of the heparin solution. Remove the 30G needle from the 3-way and remove the bubbles. Withdraw fluid in the 3-way back into the 1cc syringe to remove any bubbles that are trapped in the 3-way and reattach the tubing to the 3-way. Approximately 1cc of the mixture should remain in the syringe. The mouse will receive about 0.05cc of this mixture (as a result of flushing the catheter to maintain patency upon insertion) equaling about 1U heparin/mouse. Place this completed catheter onto the sterile field dressing with the surgical instruments.

To set-up the left leg murine catheter follow the same procedure as described above with the exception of the 3-way stopcock. The left leg is used to draw the blood and the 3-way stopcock is not required. Fill another sterile 1cc syringe with 0.15-0.2cc of the heparinized saline mixture. Hook up the 30G needle and PE-10 tubing directly to the 1cc syringe. Fill this left leg catheter system with the solution. Remove the bubbles from this system, too. Place the completed catheter onto the sterile field dressing with the sterile instruments.

2. Transducer Set-up.

Hook up a sterile transducer to the digi-med BPA 400 analyzer according to the micro-med specifications. Attach a 3-way stopcock to both ends of the transducer. Fill a 10cc syringe with Lactated Ringer solution (LR) and attach it to the 3-way so the transducer will lie flat on the benchtop. Insert a 23G needle into both ends of the piece of precut presterilized 18-inches PE-50 tubing. Attach one end of the PE-50 tubing to the 3-way with the 10cc syringe attached. Fill the 3-way and PE-50 set-up with LR. Make sure to get ALL the AIR BUBBLES OUT of the system as described in the previous section. Reattach the 3-way to the transducer and fill the transducer and 2nd 3-way with LR. Finally, attach the metal male-male leur-lock stopcock to the 23G needle of the PE-50 tubing for attachment to the right leg murine catheter.

*It is critical that fluid remain in the transducer when in operation.

*Follow calibration and zero procedures according to Micro-med protocol.

3. Surgical and Experimental Procedures:

1. Surgical Procedures.

Begin by administering an intraperitoneal injection of Pentobarbitol Sodium (Nembutol) (70mg/kg @ 1:10 dilution). This procedure is accomplished by, first, picking the mouse up from its cage using the base (most proximal end) of its tail. Next, place the animal on top of the cage while still holding its tail. Grab the neck scruff of the mouse with the thumb and middle finger on either side of the mouse just behind the forepaws. The index finger is used to pull the skin on the head/neck region back toward the scruff to immobilize the head. The mouse's tail is then wrapped and held between the little finger and the ring finger while the ring finger is pressed into the lumbar region of the mouse's spine. The mouse should be asleep within 5 minutes. After the animal is anesthetized place them onto the metal surgical plate in the supine position. The loose loop tape technique is used to immobilize the animals by taping their extremities. The loose loop technique entails simply cutting thin strips of tape and wrapping the tape loosely around each of the fore limbs inferior to the paw and around each of the hind limbs inferior to the paw. The tape is then stuck back to itself and the left over tape is attached to the board. This allows the extremities of the mice to assume a more natural anatomical position. The animal's abdominal and inguinal areas are then shaved using Oster A5 clippers size 40 blade. A 4x4 gauze is doused with betadine and the surgical area is then wiped for sterility.

After immobilization and sterilization, a nose cone with 1cc of isoflurane is placed over the mouse's nose for a few seconds before making the initial incision. The nose cone consists of a 50cc conical tube filled with gauze. Half of the bottom of the tube is cut out creating a space for the mouse's nose to rest inside without contact. A cap (bottom of a tissue storage container) is placed onto the end of the 50cc conical to ensure the isoflurane vapors don't escape. Once the animal's respirations begin to slow, a small 4-5mm incision is made in the skin parallel to the left internal oblique muscle of the abdomen and the left transverse abdominus muscle. Dissection of the femoral vein and artery follows. Make sure **not** to damage the surrounding muscles or touch nerves. To begin this dissection, separate the adipose tissue from the oblique and transverse abdominal muscles by grabbing the adipose tissue with the dumonts at the abdominal connection. Pull this tissue away from the muscle wall.

Then, blunt dissect along the abdominal muscles teasing away fascia and adipose tissue using the other pair of dumonts. Just beneath this adipose tissue lie the femoral vein and artery along with the femoral nerve.

*Be sure not to damage the vastus intermedius, medialis, and lateralis muscle of the quadriceps femoris or the rectus femoris. There is really no need to grab or even touch these muscles.

*DO NOT TOUCH the FEMORAL NERVE

Dissect away the nerve by grabbing the adipose tissue that lies next to it. Pull this tissue laterally from the vein and artery and the nerve will follow as it is embedded in this tissue. As the nerve is pulled laterally, blunt dissect the fascia by placing the other dumonts, point down, against the artery and opening and closing them. The vessels are very superficial so be sure not to dig into the underlying muscles. After the nerve is separated, use the dumonts to separate the fascia holding the vessels to the muscles. Keep the dumonts closed and slip the dumonts dorsal to the vessels. As the tip of the dumonts appears on the other side of the vein, open them to blunt dissect the fascia. Keep the dumonts dorsal to the vessel and grab the first suture. Put the suture into the dumonts and pull the suture back through the opening made between the vessels and the underlying muscles. Again, there is no need to damage any surrounding muscle.

Put a total of 3, 6-0 sutures around the vein and artery. Suture 1 is the most proximal to the abdominal muscles. Tie a knot but leave it loose and hemostat it off. The concave edge of the hemostat should rest on the animal's abdominal cavity. Suture 2 is the most distal in location. This suture can be tied off immediately ligating the vessels and hemostated, again concave side down. The distal and proximal sutures are used to pull the vessels taut (to prevent blood loss) and lift them a bit to aid in catheter insertion. Suture 3 is a catheter support suture. Place this suture between the distal and proximal sutures. Tie a loose knot that will be used to secure the catheter inside the vessel after insertion. After the sutures are secure, identify the artery by the thick vessel wall. It is very white. Make a small incision on the top of the artery using the microscissors. Make this hole close to the distal suture so there is an ample amount of artery for the initial catheter insertion. Use the dumonts to open the hole by placing one end of the dumonts into the arterial vessel lumen and closing them over the vessel wall. Be sure the middle suture is proximal to the arterial hole so it can be used to hold the catheter in place after initial insertion. While holding the arterial wall push the catheter into the lumen while pulling the vessel over the catheter. Lightly tie down the middle support suture to hold the catheter in place. Release the proximal hemostat. This release will loosen the proximal suture and reopen the suture around the vessels. At this point, the arterial pressure should push blood back into the catheter. Pulsating blood should be visible in the catheter. Hold the vessels around the catheter with one dumont and use the other to push the catheter into the vessel about 4-5mm. Holding the vessel around the catheter helps to prevent tearing of the artery. The tip of the catheter should rest just beneath the internal oblique and transverse abdominus muscles. For blood clot prevention in the arterial line, withdraw blood into the catheter and push it back into the mouse several times to infuse heparinized fluid. Repeat this procedure for the other leg for bilateral femoral artery cannulation. Hook the animal up to the physiological parameter monitor, i.e. the BPA-400 analyzer and flush the arterial lines. Put 1 or 2 drops of sterile saline into the surgical opening to keep the surrounding tissue moist. Be sure to keep this area saturated throughout the procedure. Place a sterile field dressing over the animal throughout the procedure to help maintain sterility.

Place surgical instruments into 70% alcohol and wipe them with sterile gauze. Put them into the hot bead sterilizer for ~20 seconds for sterilization between animals. Remove the surgical instruments and spray them with 70% alcohol to help them cool off. Place them onto the sterile pad. Make sure there is no alcohol left on the instruments that will drip back into the next animals.

2. Hemorrhagic Shock.

Over a 15min. timeframe, approximately 1/2 of the mouse's blood volume is withdrawn achieving a mean arterial pressure of 28-32mm Hg.

*For a 25-27g mouse the initial volume of blood withdrawn to achieve the desired pressure is approximately 0.6cc

This procedure is a fixed pressure method as opposed to a fixed volume. These procedures can, however be followed for both fixed pressure and fixed volume hemorrhage. While being consistently monitored, the animal will remain in hemorrhagic shock for 1.5-3hours. As the animal attempts to compensate and the mean arterial pressure begins to rise again slightly (visible via the BP/HR analyzer) withdraw more blood to achieve the desired pressure. Although supplements (0.05cc Nembutol IP) are rarely ever needed during the HS procedure, animal respiration, whisker movement, reflex tests, and the digital BP/HR reading will help determine when an animal needs a supplement of anesthesia. Animals are kept under lamp and on a circulating heating pad to help maintain a temperature of 36-37°C through the Hemorrhagic Shock procedure. Temperature is checked via a rectal probe.

3. Resuscitation.

After the shock time elapses, the animal is resuscitated (R) using Lactated Ringers (LR) solution at a fixed volume of 3x each animal's shed blood volume. The LR volume is administered via a syringe pump set to dispense, at a constant rate, the volume over a 15min. interval. Remove the catheter and ligate the vessels using the 3 sutures. Pull the catheter just past the proximal suture and tie this suture completely off. That will prevent any blood loss. Collateral flow prevents the hind limbs from becoming ischemic.

4. Post Rescitation.

After R both hind limb openings are sewn up using sterile 4-0 PDSII suture. The loose loop tape is removed and the animals are placed into a clean cage which is kept on a circulating heating pad for several hours post recovery. Analgesic must be administered as the animals begin to waken from the anesthesia to properly manage pain. Buprenorphine (0.1mg/kg) is injected subcutaneously as the animals begin physical activity, but not before, so as not to compromise respiratory function. Use of aggressive post operative monitoring as warranted by IACUC approved protocol, individual animal behaviors, and current medical conditions is recommended.

4. Secrets for Success:

1. When dissecting the vessels, Be sure NOT to TOUCH the Femoral Nerve

2. Be sure not to damage the rectus femoris, rectus lateralis, and vastus medialis muscles. There is really no need to grab or even touch these muscles.
3. Do Not make the angle of the bevel of the catheter too sharp as this will lead to the catheter exiting the lumen and uncontrolled bleeding
4. Make sure to take the extra effort to remain as sterile as possible.
5. Make sure there are NO air bubbles in any of the catheter or transducer systems.
6. Make sure to have LR fluid in transducer while it is in operation. A dry run could damage your BP system.
7. Make the arterial hole small.
8. Make the arterial hole close to the distal suture.
9. Push catheter while simultaneously pulling vessel over it for catheter insertion in order to avoid ripping the artery.
10. Be sure to keep the surgical openings in the legs saturated with sterile saline to prevent uncontrolled tissue damage or fluid loss.
11. Patience and finesse are critical.
12. Pay close attention to the animal's physiology throughout the experiment.

5. Post Operative Concerns:

1. Check for ischemia in leg. Although collateral flow should prevent this.
2. Animal could have trouble using hind limbs as a result of surgical manipulation and associated inflammation. Manage pain appropriately.
3. Touching and subsequently damaging the nerve could lead to inability of the animal to mobilize.

Disclosures

Experiments on animals were overseen and performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee and the Research Conduct and Compliance Office of the University of Pittsburgh, a fully accredited AALAS/AALAC institution. Animal sources include Jackson Laboratories and Charles Rivers Laboratories. All animals undergo extensive health assurance through each vendor as well as the University of Pittsburgh's internal animal health monitoring programs. This research is conducted in accordance with the US government principals for Use of vertebrate animals. The program is registered with the USDA, and has a letter of assurance with the Public Health Service Office of Laboratory Animal Welfare.

Acknowledgements

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