Video Article

Pseudofracture: An Acute Peripheral Tissue Trauma Model

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Abstract

Following trauma there is an early hyper-reactive inflammatory response that can lead to multiple organ dysfunction and high mortality in trauma patients; this response is often accompanied by a delayed immunosuppression that adds the clinical complications of infection and can also increase mortality.[1-9] Many studies have begun to assess these changes in the reactivity of the immune system following trauma.[10-15] Immunologic studies are greatly supported through the wide variety of transgenic and knockout mice available for *in vivo* modeling; these strains aid in detailed investigations to assess the molecular pathways involved in the immunologic responses.[16-21]

The challenge in experimental murine trauma modeling is long term investigation, as fracture fixation techniques in mice, can be complex and not easily reproducible.[22-30]

This pseudofracture model, an easily reproduced trauma model, overcomes these difficulties by immunologically mimicking an extremity fracture environment, while allowing freedom of movement in the animals and long term survival without the continual, prolonged use of anaesthesia. The intent is to recreate the features of long bone fracture; injured muscle and soft tissue are exposed to damaged bone and bone marrow without breaking the native bone.

The pseudofracture model consists of two parts: a bilateral muscle crush injury to the hindlimbs, followed by injection of a bone solution into these injured muscles. The bone solution is prepared by harvesting the long bones from both hindlimbs of an age- and weight-matched syngeneic donor. These bones are then crushed and resuspended in phosphate buffered saline to create the bone solution.

Bilateral femur fracture is a commonly used and well-established model of extremity trauma, and was the comparative model during the development of the pseudofracture model. Among the variety of available fracture models, we chose to use a closed method of fracture with soft tissue injury as our comparison to the pseudofracture, as we wanted a sterile yet proportionally severe peripheral tissue trauma model. [31]

Hemorrhagic shock is a common finding in the setting of severe trauma, and the global hypoperfusion adds a very relevant element to a trauma model. [32-36] The pseudofracture model can be easily combined with a hemorrhagic shock model for a multiple trauma model of high severity. [37]

Protocol

1. Instrument and Surgical Field Preparation:

All experimental procedures are performed using aseptic techniques. Before beginning, the experimental area must be thoroughly cleaned and sterilized. The benchtop should be disinfected, allowed to air dry and then wiped down with 70% alcohol. Place a surgical blue pad and sterile field dressing in the experimental work area.

All materials and instruments are autoclave sterilized before use. Syringes and needles are received sterile. The researcher should be appropriately garbed in lab coat, surgical mask, and sterile surgical gloves.

Our surgical instruments are autoclave sterilized each evening. They are washed after surgery using antibacterial soap and water. They are allowed to dry on a clean surgical blue pad. They are then carefully placed into a sterilization pouch and sterilized for later use.

Turn on the hot bead sterilizer to ensure it reaches the appropriate temperature - 300-350°F before starting the experiment. This sterilizer will be used to clean instruments between experimental procedures on respective mice. Get a stainless steel instrument tray and fill it 1/3 of the way with 70% ethanol. There should be enough 70% Ethanol to cover your surgical instruments. Turn on the circulating heating pad and place a surgical blue pad over it and then a sterile field dressing on top of that. This heater is used to ensure adequate warmth of the mouse during the experimental procedure and recovery period. Place all sterile instruments, gauze, syringes, needles and a 1.5mL microfuge tube on the sterile field dressing. Be careful when opening any sterile instruments and other items, do not to contaminate them by touching them. It is best to use sterile gloves when doing this setup procedure.

2. Pseudofracture Model - Experimental Procedures:

1. Induction of anaesthesia and positioning of the mouse.

Begin by administering an intraperitoneal injection of Pentobarbitol (70mg/kg). This is accomplished by first lifting the mouse from its cage using the proximal end of its tail. The mouse must then be weighed to calculate the correct dosage of anaesthesic. Next, place the animal on top of the cage while still holding its tail. Grab the scruff of the neck 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 and partially immobilize the head. The mouse's tail is held

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between the little finger and the ring finger while the ring finger is pressed into the lumbar region of the mouse's spine. The anesthesia should take full effect within approximately 5 minutes, and should be confirmed with the reflex test.

After the animal is asleep, place the mouse in the supine position onto a plexiglass board and use a loose loop tape technique to immobilize them by taping their extremities. The loose loop technique simply entails cutting thin strips of tape and wrapping the tape loosely around the distal portion of each extremity. The tape is then stuck back to itself and the left over tape is attached to the board. This allows the experimental manipulation to be performed in a consistent way as well as ensuring the extremities of the mice assume a more natural position. The animal's abdominal, inguinal, and thigh regions are then shaved using our Oster A5 clippers (size 40 blade), to help maintain a sterile field. A 4x4 gauze is doused with betadine and the surgical area is then wiped to sterilize. A sterile field dressing is then folded and draped across the mouse to allow exposure of only the lower extremities and head.

After immobilization and sterilization, place a nose cone with isoflurane over the mouse's nose for a few seconds before beginning the experimental procedure. The nose cone consists of a 50cc conical tube filled with gauze. Half of the bottom end of the tube is cut out creating a space for the mouse's nose to fit into. A cap (bottom of a tissue storage container i.e. straight side wide mouth jar) is placed onto the cut end of the conical to ensure the isoflurane vapors remain in the tube when not in use. One mL of isoflurane should be added to the gauze within the cone. Meticulous attention should be paid to the mouse during use of anaesthesia and particularly use of isoflurane via the nose cone. Once the animal's respirations begin to slow, the experiment can begin, and the nose cone should be removed and closed.

If additional anesthesia is required throughout any part of the experiment, additional pentobarbital may be injected or isoflurane administered. Alternative methods of isofluorane administration are available and updated guidelines recommend the use of a precision vaporizer to allow better control of anesthesia, if feasible. However, close attention to the respiratory physiology of the mouse must be guaranteed with any of these methods.

2. Pseudofracture experiment.

Pseudo-fracture is a combination of soft tissue injury followed by bone solution injection to the injured muscle and is performed bilaterally to the hindlimbs. The bone solution must be prepared before any experimental manipulation of the recipient mice begins.

3. Bone solution preparation.

The bone solution prepared from one donor mouse will be enough for 3 recipient mice. A syngeneic donor mouse should be used that is age- and weight-matched to the recipient experimental mouse.

The donor mouse will be euthanized with inhaled isoflurane. The donor will then be taped to the plexiglass board with careful attention to tape the lower extremities only at the tip of the foot. The lower extremities must be carefully shaven and thoroughly covered in betadine, and then wiped with alcohol for sterility. Then the femur and tibia will be surgically removed in a sterile manner from both lower extremities. To remove the long bones from the lower extremity, make a surgical incision into the skin in the inguinal region, and continue to cut the skin along the length of the extremity, down to the ankle. Retract the skin, and dissect it away from the subcutaneous fascia and muscle both on the medial and lateral aspects for maximum exposure. Insert one blade of the scissors under the muscles that lie anterior and lateral to the tibia (tibialis anterior, extensor digitorum longus, extensor hallicus longus, peroneus longus, peroneus brevis) and slide the blade proximally and distally to lift the muscles cleanly away from the underlying bone. Repeat this technique on the posterior and medial underside of the tibia to separate the muscles underneath the tibia (gastrocnemius, soleus, plantaris, tibialis posterior, flexor digitorum longus, flexor hallicus longus). A minimal additional exertion should be used on the posterior side when sliding distally, in order to snap the fibula away from the tibia. At the proximal end of these leg muscles cut the tendons as close to their insertions as possible and retract the muscles distally, while pulling on the muscle group together in downward fashion a small tug will release the distal attachments of these muscles from the ankle joint. The ankle joint can be cut through directly with the scissors. Do not detach the tibia from the femur at this point! This connection gives additional leverage that will aid in the dissection of the femur from the hip joint. Follow a similar technique for release of the muscles from the femur and perform this posteriorly and both medially and laterally also if needed. Cut the distal attachment of these muscle groups to release them from the knee joint. Continue the dissection and follow underneath the muscles to dissect the end of the femur out of the hip joint. To aid in this dissection, the skin incision should be extended as far as required to achieve adequate exposure. Motion of the femur at the hip joint can help locate the proximal end of the femur during dissection.

Once the femur (with tibia attached) is removed from the donor mouse place them on a sterile 4x4gauze. The two bones can be separated at the knee joint by simply grasping each bone in a separate hand and gently twisting/rotating the two bones in opposite directions along their long axis. Gentle dissection and handling of these two bones is recommended as they can be easily fractured with any over-exertion of the manual manipulation. To remove the remaining tendon attachments from the long bones, use a dry sterile piece of 4x4 gauze wrapped around the length of the bone. Grasp this gauze tightly against the bone an pull it the full length of the bone several times, this will scrape the remaining tissues off the surface of the bone as they will adhere to the gauze and be quickly and easily pulled away from the bones. The collected bones should then be placed directly into a sterile 1.5mL microfuge tube and placed on ice for transport.

These four donor bones will be taken to a biological safety cabinet for preparation for the bone solution. The hood must be thoroughly disinfected before use, place a sterile field dressing over the work area within the hood. Place a sterile mortar and pestle, sterile 8mL tube and sterile Phosphate Buffered Saline (PBS) in the work area. A 1mL pipetteman, and respective tips should be available within the hood.

A sterile pair of forceps should be used to remove the harvested bones in a sterile fashion from the 1.5mL microfuge tube and placed into the mortar; the pestle will then be used to gently crush the bones. Phosphate Buffered Saline is the vehicle used to resuspend the bone fragments for injection. Pipette 1mL of PBS into the mortar and continue to crush the remaining fragments, with additional circular motions to ensure full resuspension. Then add another 1mL of PBS and continue crushing to create the 'bone solution'. The solution should have a pink hue, and there will be remnants left at the bottom of the mortar that cannot be fully broken up. Slowly pour the solution out of the mortar into the 8mL tube, to catch the maximum volume, while ensuring the larger remnants remain behind in the mortar. This bone solution should remain on ice until its experimental use and will be transferred to 1mL syringes for injection. A twenty gauge needle will be later attached to the 1mL syringe containing the bone solution in order to administer it to the mouse. This large needle size was chosen to ensure that all the bone fragments enter the region upon injection and don't block the needle.

Before injection to recipient mouse, a drop of the bone solution is placed on a MacConkey agar plate for culture (incubation for 24-48hours). This is to ensure sterility of the bone solution. The pH of the bone solution also must be checked to ensure a neutral pH.

4. Soft tissue injury.

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This soft tissue injury is a bilateral hindlimb crush injury. The initial positioning of the mouse, with hind limbs slightly abducted and laterally rotated, helps in accessibility to the correct muscle group, the knee flexors (biceps femoris, semitendinosus and semimembranosus muscles). A large 18cm hemostat will be used to perform the crush injury. The force distribution of these applied hemostats was analyzed and found to be 270psi using Topaq Pressure Analysis System by Sensor Products Inc. The hemostat must be clamped around the posterior thigh musculature, midpoint along the femur, with its convex curve facing the femur. The hemostat should then be locked shut to the first click only, and remain for 30 seconds. Careful attention to the placement of this hemostat is important - it must not be clamped over the femur, to ensure it is not fractured. This is should be performed in a consistent manner each time to guarantee a reproducible injury between mice. This crush injury is performed on both lower extremities.

5. Bone solution injection.

The recipient experimental mouse will be anesthetized and prepared as previously described, and will have undergone soft tissue injury prior to this bone injection.

The bone solution will then be injected into the crushed thigh musculature of the recipient mouse bilaterally. Using a 20 gauge needle, 0.15mL of this solution will be injected into the posterior muscles of each thigh. Enter the needle through the skin approximately 2~3mm and pay attention to the distance of insertion of the needle - you will feel as the tip of the bevel just touches against the femur, this position is ideal. Inject the bone solution now. Withdraw the needle and quickly place a sterile gloved finger over the injection site to stop any back flow of bone solution out of the wound. Hold this finger there for several seconds.

The mouse will then be placed back into the cage and allowed full freedom of movement directly as anaesthesia subsides. Appropriate pain management must be administered as the mouse awakens.

6. Post-operative/recovery period.

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. Appropriate warmth should be ensured with an additional heat lamp if required.

Food and water will be readily available.
Analgesic must be administered as the animals begin to waken from the anesthesia in order to correctly 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.

Mice should be constantly monitored during anaesthesia, until recovery from anaesthesia or experimental endpoint. Mice should be also be very carefully monitored in the postoperative period and any additional pain medication required should be administered as needed. Monitor animal physical activity, respiratory status, food and water intake and any signs of distress (labored breathing, pain, changes in eating and drinking habits) should be addressed accordingly.

Place your surgical instruments into 70% alcohol and wipe them with sterile gauze then put them into the microbead 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 field dressing. Make sure there is no alcohol left on the instruments that will drip back into the next animals.

3. Comparative Bilateral Femur Fracture Model - Experimental Procedures:

The experimental mouse will be anesthetized and prepared as initially described. A large 18cm hemostat should be clamped around the hindlimb approximately 2-3mm above the knee joint with its convex surface facing the knee. Place your thumb on top of the clamped hemostat and your index finger between the hemostat and hip. Be sure to feel the femur before twisting so you know where you are breaking. Then twist hemostat counterclockwise while twisting your other hand clockwise.
Repeat procedure with contralateral leg.

4. Multiple Trauma Model - Experimental Procedure for Combination of Pseudofracture with Hemorrhagic Shock:

Recipient mouse will be anesthetized and prepared as initially described. Mice that are to undergo hemorrhagic shock in combination with pseudo-fracture will have femoral artery cannulation before the pseudofracture procedure and then will be hemorrhaged from this catheter once the pseudo-fracture has been completed.

5. Secrets for Success:

General:

- Make sure you take the extra effort to remain as sterile as possible.
- Meticulous attention should be paid to the overall physiological condition of the mouse, including respiratory rate, during administration of anesthesia and particularly isoflurane via the nose cone.

Bone solution preparation:

- Gentle manipulation of the bones - as can be easily fractured with over-exertion during simple manual manipulation!
- Fibula removal from the tibia should be ensured upon dissection.
- Begin by crushing bones gently at first - so as to keep all the fragments within the mortar!
- Add only 1 mL PBS initially and continue crushing the remaining bone fragments, once a good suspension has formed, add the remaining 1mL.
- Only the solution from the mortar and pestle should be collected - without the larger remaining tissues that could not be fully broken down - these be may block/get lodged in the needle upon injection into the recipient mouse.

Soft tissue injury:

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- Careful placement of this hemostat is important - not over the femur or it would be fractured. Clamp the hemostat over the correct musculature (posterior thigh - knee flexors) for reproducible injury.

Bone solution injection:

- When injecting the solution, pay attention the distance of insertion of the needle - you will feel as the tip of the bevel just touches against the femur, this position is ideal.
- Withdraw the needle and quickly place a finger over the injection site to stop any back flow of bone solution out of the wound. Hold finger there for several seconds.

6. Post Operative Concerns:

- Check for infection in the leg.
- Check bone solution culture.
- Animal could have trouble using hind limbs as a result of experimental manipulation and associated inflammation. Manage pain appropriately.

Discussion

Pseudofracture, a reproducible murine model of sterile musculoskeletal trauma, allows for evaluation of post-traumatic immune responses. The pseudofracture model immunologically mimics an extremity fracture environment through recreation of the features of a long bone fracture: injured muscle and soft tissue are exposed to damaged bone and bone marrow without breaking the native bone.[38,39] A biphasic immune response can be seen following pseudofracture trauma which consists of an early hyperinflammatory response that can be seen to peak at 6 hours followed by a second component of delayed immunosuppression depicted as a trough around 48hrs. This model helps to overcome some of the challenges of experimental murine trauma modeling such as fracture-fixation which can be complex and not easily reproducible. In particular this model permits late term study of post-traumatic immune responses as it allows for long term survival in the animals without fracture of the native bone.

Disclosures

Experiments on animals were 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, an AALAS/AAALAC accredited 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.

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