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Contusion Spinal Cord Injury via a Microsurgical Laminectomy in the Regenerative Axolotl

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Abstract

The purpose of this study is to establish a standardized and reproducible regenerative blunt spinal cord injury model in the axolotl (Ambystoma mexicanum). Most clinical spinal cord injuries occur as high energy blunt traumas, inducing contusion injuries. However, most studies in the axolotl spinal cord have been conducted with sharp traumas. Hence, this study aims to produce a more clinically relevant regenerative model. Due to their impressive ability to regenerate almost any tissue, axolotls are widely used as models in regenerative studies and have been used extensively in spinal cord injury (SCI) studies. In this protocol, the axolotls are anesthetized by submersion in a benzocaine solution. Under the microscope, an angular incision is made bilaterally at a level just caudal to the hind limbs. From this incision, it is possible to dissect and expose the spinous processes. Using forceps and scissors, a two-level laminectomy is performed, exposing the spinal cord. A custom trauma device consisting of a falling rod in a cylinder is constructed, and this device is used to induce a contusion injury to the spinal cord. The incisions are then sutured, and the animal recovers from anesthesia. The surgical approach is successful in exposing the spinal cord. The trauma mechanism can produce contusion injuries to the spinal cord, as confirmed by histology, MRI, and neurological examination. Finally, the spinal cord regenerates from the injury. The critical step of the protocol is removing the spinous processes without inflicting damage to the spinal cord. This step requires training to ensure a safe procedure. Furthermore, wound closure is highly dependent on not inflicting unnecessary damage to the skin during incision. The protocol was performed in a randomized study of 12 animals.

Video Link

The video component of this article can be found at https://www.jove.com/video/60337/

Introduction

The overall goal of this study was to establish a controlled and reproducible microsurgical method for inflicting blunt and sharp SCI to the axolotl (Ambystoma mexicanum), producing a regenerative spinal cord injury model.

SCI is a severe condition that, depending on the level and extent, inflicts neurological disability to the extremities along with impaired bladder and bowel control1,2,3. Most SCI are the result of high energy blunt trauma such as traffic accidents and falls4,5. Sharp injuries are very rare. Therefore, the most common macroscopic injury type is contusions.

The mammalian central nervous system (CNS) is a non-regenerative tissue, hence no restoration of neurological tissue following SCI is seen6,7,8. On the other hand, some animals have an intriguing ability to regenerate tissues, including CNS tissue. One of these animals is the axolotl. It is widely used in studies of regenerative biology and is of interest in spinal cord regeneration, because it is a vertebrate9,10,11,12.

Most SCI studies in the axolotl are performed as either amputation of the entire tail or ablation of a larger part of the spinal cord9,10,11,12. Recently, a new study was published on blunt injuries13 that mimics clinical situations better. Whereas complete appendage amputation in the axolotl results in full regeneration, some non-amputation-based regenerative phenomena are dependent on the critical size defect (CSD)14,15. This means that injuries exceeding a critical threshold are not regenerated. To develop a regenerative model with a higher clinical translational value, this study investigated whether a 2 mm blunt trauma would exceed the CSD limit.

This method is relevant for researchers working on spinal cord regeneration in small animal models, especially in the axolotl. Furthermore, it may be of more general interest, because it exhibits a way of using standard laboratory equipment to develop a blunt trauma mechanism that is suitable for use in small animals in general.
Protocol

All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this study. The study was conducted under the approval id: 2015-15-0201-0061 by the Danish Animal Experiment Inspectorate. Animals were Mexican axolotls (*Ambystoma mexicanum*, mean body mass ± STD: 12.12 g ± 1.25 g).

1. Preparation

1. **Prepare axolotl for anesthesia.**
   1. Use high quality non-chemically treated tap water. If unavailable, use 40% Holtfreter’s solution.
   2. Dissolve 200 mg of ethyl 4-aminobenzoate (benzocaine) in 3 mL of acetone. Dissolve this solution in 1 L of tap water or 40% Holtfreter’s solution.

2. Use a standard Petri dish (100 mm in diameter) placed under a stereo microscope as a surgical table. Place a surgical textile cloth on the Petri dish.
   
   **NOTE:** Using a Petri dish as a surgical area enables moving and rotation of the animal without touching it, ensuring spinal stability during surgery.

3. Prepare all sterile microsurgical instruments (i.e., scissors and anatomical forceps).

2. Anesthesia

1. Place the axolotl in a container with benzocaine solution for approximately 45 min to ensure deep and stable anesthesia.
   
   **NOTE:** The given concentration of benzocaine will cause anesthesia in all sizes of axolotls.

2. Check for signs of general anesthesia within 30-45 min. These include a complete lack of gill movements, righting reflex, or response to either tactile or painful stimuli (gentle pinching of toe web).

3. To maintain anesthesia, wrap the animals in paper towels wetted in the anesthetic solution. Wet these regularly with this solution during the surgical procedure to ensure that the skin and gills are kept moist.

4. Recover the animal after the surgery by placing it in a container containing fresh tap water. Observe signs of recovery, such as gill movement and regained righting reflex, within 1 h.

3. Microsurgical Laminectomy

   **NOTE:** The laminectomy is performed under a stereomicroscope.

1. Place the animal in the prone position on the Petri dish. Wrap it in paper towels so that the tail is exposed.
   
   **NOTE:** The paper towels are excellent for ensuring stability throughout the procedure.

2. Identify the hind limbs. Make the first incision just caudal to them.
   1. With a pair of microscissors, perform a vertical incision from the keel until the bony prominence of the spinous processes are felt.
     
     **NOTE:** Be very careful when grasping the keel and skin with forceps, because these easily inflict damage to the delicate skin.
   2. Extend the cut laterally, so the incision traverses the entire width of the tail.
   3. Grasp the spinous process with forceps to ensure the right depth.
   4. Extend the vertical incisions 1 mm below the spinous process on both sides.

3. Place the animal on one side to perform ventral and horizontal incisions as stated below.
   1. With a pair of microscissors, starting from the ventral point of the vertical incision, make a horizontal incision of approximately 15 mm for animals 10-20 g in weight. Make the incision longer for larger animals, and shorter for smaller animals.
   2. Using the scissors, dissect medially through the horizontal incision until the vertebral column is felt in the midline.
   3. Repeat steps 3.3, 3.3.1, and 3.3.2 on the other side of the animal.

4. Having dissected in the deep medial plane from both sides, dissect through the midline, thereby connecting the two horizontal incisions.
   1. Move the free piece of tail and keel to one side, exposing the spinous processes (Figure 1).
   2. Fixate the tail piece using wet paper towels.

5. Place the animal in the prone position again with the head facing the surgeon’s non-dominant side.
   1. With a pair of forceps, grasp the spinous processes just caudal to the hind limbs. Apply a gentle lift both up and towards the head of the animal.
   2. Place the blades of a pair of microscissors horizontal around the process and gently cut it. The lift on the process ensures that it is now removed, exposing the spinal cord.
   3. Grasp the spinous process just caudal to the one that was just removed and repeat steps 3.5.1 and 3.5.2.
     
     **NOTE:** This should leave an exposed spinal cord corresponding to two vertebral levels. When performing the laminectomy, a white foamy secretion often appears. The spinal cord is easily identified by its distinctive shine, along with a vessel running along the midline.
   4. Depending on the size of the animal, the exposed area may not be wide enough. Using two pairs of forceps, grasp the laminae on both sides of the spinal cord and twist these laterally with a gentle movement.
4. Introducing a Contusion Type Injury (Figure 2)

1. Keep the animal in the prone position.
2. Use the Petri dish to transfer the animal to the trauma unit.
3. Have an assistant shine a flashlight on the spinal cord.
4. Place the contusion trauma unit cylinder above the exposed spinal cord using the microadjusters on the unit. Aim through the cylinder.
5. Lower the cylinder until it is level with the laminae.
6. Attach the falling rod to the electromagnet. Place the desired falling height adjustment cylinder on the trauma unit.
7. Place the falling rod in the cylinder.
   
   NOTE: For a blinded study, the surgeon should now leave the room without knowing if the animal will be assigned to an injury or a sham surgery group.
8. Turn off the electromagnet. The rod falls to the exposed spinal cord.
9. Use the height adjustment screw to lift the rod from the spinal cord.
10. Confirm the injury by looking at the spinal cord through the microscope. The injured site will appear darker, and bleeding from the midline vessel will be apparent.

5. Introducing a Sharp Injury

   NOTE: Perform these steps after 3.5.4.

1. With a pair of microscissors cut the spinal cord in a perfect vertical cut.
2. Repeat the cut 2 mm to the caudal side of the body.
   
   NOTE: The length of the removed piece of spinal cord can be adjusted as per the study requirement. However, a 2 mm cut will be regenerable.
3. Ensure that the cuts are complete. Upon completion, feel the blades of the scissors scraping along the ventral part of the spinal canal.
4. Lift the 2 mm piece of spinal cord from the spinal canal.

6. Closing the Surgical Wound

1. Return the animal to the surgical table. In a blinded study, reposition the keel so the spinal cord is not visible to the surgeon.
2. Keep the animal in the prone position.
   
   1. Begin placing 10.0 nylon sutures from the most caudal part of the horizontal incision. Close the wounds in one layer.
   
   NOTE: Do not grasp the skin too tight, because it will inflict necrosis.
   2. Work towards the vertical part of the incision.
   3. When reaching the angle, turn the Petri dish and suture the other horizontal incision.
   4. Set sutures on the vertical incisions.
   5. Do not place sutures in the uppermost part of the keel, because the skin here will not be able to hold.

7. Returning the Animal to the Anesthetic-free Solution

1. Lift the Petri dish with the animal and submerge both very gently into fresh water only 5 cm deep and let the animal slide off.
   
   NOTE: The shallow water depth ensures that the animal will not attempt to swim to the surface to breathe.
2. Do not change the water during the first week.
3. When feeding the animals, ensure that the food is placed near the animal’s head.
   
   NOTE: The purpose of these measures is to avoid as much movement as possible during the first week.

8. Postoperative Ultrasound

1. Prior to the termination of anesthesia, use a high frequency ultrasound system to acquire images of the injury that can be used for the construction of three-dimensional images of the SCI site.
2. Attach the transducer to a micromanipulator preferably governed by a remote joystick.
3. Submerge the anesthetized animal in the prone position into a small container filled with anesthetic solution.
   
   NOTE: Fix the animal with miniature sandbags or other equipment to avoid movement during the scanning sequence.
4. Align the tip of the transducer with the animal’s length axis and submerge it into the benzocaine solution until it is only a few millimeters above the keel behind the hind limbs of the animal.
5. Identify the SCI site.
   
   NOTE: The injury site is easily recognizable due to the missing spinous processes directly above the SCI.
6. Optimize the image by adjusting the ultrasound settings. Ensure that the SCI site is in the center of the image. Adjust the field of view (i.e., image depth, depth offset, and image width) to cover the SCI site and adjacent healthy tissue. Adjust the two-dimensional gain to optimize the image contrast.
7. By sweeping the ultrasound transducer across the SCI site with an electronically operated micromanipulator, acquire B-mode images covering the SCI site at multiple sagittal cross-sectional slice locations, with consecutive slices with an interslice interval of 50 µm. Acquire cine-images containing 500 frames with a frame rate of ~50 frames/s and a transducer frequency of 40 MHz.
   
   NOTE: This setup requires an electronic micromanipulator governed by a remote joystick (step 8.2).
8. After finishing the scanning sequence return to step 7.
Representative Results

The purpose of the protocol is to produce an SCI that will paralyze the motor and sensory functions caudal to the injury. Because the axolotl is regeneration-competent it restores function within weeks, allowing researchers to study CNS regeneration during a short time span.

Anesthesia was provided for 45 min to all animals, and no episodes of preterm recovery were experienced. All animals recovered within an hour and showed no signs of damage from anesthesia in the following weeks.\(^{13,16}\)

The laminectomy was successful in all animals. However, anatomical variation in the width of the spinal canal called for the widening of the canal using forceps and a twist in some individuals. Furthermore, residual laminae in some individuals prevented the falling rod from reaching its target, hence making it imperative that the surgeon clean the field from the residual bone and prominences.

Closing the incisions was associated with some difficulties, especially during the piloting phase of the study. Sutures in the top part of the keel would not hold and resulted in insufficient closures. The closure of one animal in the study did not hold, resulting in the keel being torn, subsequent infection, and death. This stresses the need for careful suturing along the entire incisions.

The initial mechanical injuries were obvious during the procedure. During the model development, injured and sham animals were stained with hematoxylin and eosin to validate the injury. Representative results of each group are shown in Figure 3A1, A2 and Figure 3C1, C2. Regeneration was confirmed by histological sections preparations made after nine weeks (Figure 3B1, B2 and Figure 3D1, D2), which showed a reestablished spinal cord connection in the SCI animals.

Injury and regeneration can be followed by examining neurological function. Stimulating the tail with a light touch and pinching from forceps will reveal whether tactile and nociceptive sensory functions have been lost and potentially reestablished. A neurological score was defined based on the reaction of the animal: 0 point = no response, 1 point = local tail movement, 2 points = truncal movement, 3 points = coordinated movement of limbs and/or head alongside with truncal movement, 4 points = animals with immediate coordinated fast movement. In six SCI animals versus five sham animals the loss of neurological function three weeks post injury was found, and a gradual restoration within nine weeks (Figure 4 and Supplementary Video 1).

Ultrasonographic images of the injured spinal cord can be obtained using the above protocol. Visualizing the SCI site was possible due to the obvious lack of bony spinous processes (Figure 5). Furthermore, using the B-mode the dorsal artery of the uninjured spinal cord could be visualized, yielding a marker of vessel integrity.

It is possible to test the animals immediately upon reawakening. However, some animals expressed local small amplitude, repetitive, and rhythmic tail movement upon stimulation comparable to the clonus phenomena observed in human SCI. These movements might represent clonus or a lack of central reflex suppression and could potentially cause more damage to the newly injured spinal cord. Therefore, testing the animals is not recommend before one-week post injury.

From simple qualitative observation of the animals, it will be evident that the tail is paralyzed, and swimming is significantly inhibited, making the animals completely dependent on moving their limbs. These observations will also validate the success of the protocol.

High-field MRI scans (9.4 T) were performed immediately after injury to visualize the injury in vivo (Figure 6). However, the scans were generally low in signal-to-noise ratio compared to those of non-operated animals, likely due to bleeding and hemosiderin. Hence, it was concluded that MRI was a suboptimal method to validate the injury and success of the protocol.
Figure 1: Schematic drawing of the microsurgical laminectomy. Please click here to view a larger version of this figure.
Figure 2: Schematic drawing of the contusion trauma mechanism. (A) The entire setup, showing the falling rod above the animal. (B) The disassembled mechanism, showing how the rod is disconnected from the electromagnet. (C) The falling rod is connected to the electromagnet. The falling height adjustment cylinder is installed, and the electromagnet and rod loaded into the cylinder. Height adjustment of the entire system is controlled by an adjusting wheel. (D) Turning off the electromagnet will cause the rod to fall without the operator touching the system. Figure was originally published by Thygesen et al. Please click here to view a larger version of this figure.
Figure 3: Histological sections hematoxylin and eosin stained immediately and nine weeks post injury. (A1) SCI animal immediately after injury. (B1) SCI animal at nine weeks. (C1) Sham surgery animal immediately after injury. (D1) Sham animal at nine weeks. Red square = marks the injury of the SCI animals, and the laminectomy of the sham animal. Figure 2A, Figure 2B, Figure 2C are magnifications of these areas at 5x. Blue arrow = uninjured spinal cord. This figure was originally published by Thygesen et al. Please click here to view a larger version of this figure.
Figure 4: Graph of response to tactile stimuli. The response of the SCI groups is lower after three weeks, compared to the sham group. WPI = weeks post injury, Black line = SCI, Grey color = sham. Sham n = 5, SCI n = 6. Figure was originally published by Thygesen et al.13 Please click here to view a larger version of this figure.

Figure 5: Ultrasonographic image showing the spinal cord in a sagittal section. Yellow lines mark the spinal cord, yellow circle the injury site, and white arrows mark the vertebrae. Please click here to view a larger version of this figure.
Figure 6: MRI scans at different time points post injury or sham surgery. CSF surrounding the spinal cord is lacking, especially at three WPI for the SCI animal, indicating swelling of the spinal cord. Darkening of the spinal cord indicates edema as well. Notice how these changes disappear as regeneration progresses. Yellow arrow = the area of laminectomy. Figure was originally published by Thygesen et al. Please click here to view a larger version of this figure.

Supplemental Video 1: Video showing the neurological function after tactile stimuli and later a nociceptive stimulus. First a healthy control animal, and then an animal suffering from SCI. Please click here to download this video.
Discussion

Because risk of injury to the spinal cord is significant, the critical steps of the protocol are removing the spinal processes and widening of the bony access to the spinal canal if needed. As mentioned in the protocol, removing the most cranial process first is highly recommended. This will mean that the more caudal processes protect the spinal cord from being hit by the scissors. It is recommended to ensure enough surgical access, meaning to not make too small a primary incision. Also, when grasping anything with forceps, the direction of the pull applied must always be considered. Applying a gentle pull away from the spinal cord will protect it in the event of the grasp failing and a slip of the instrument.

The surgical procedure in the axolotl is not different from other animals. However, certain important differences do exist, primarily attributable to the tissue composition and size of the animal. The axolotl keel skin is very fragile, and paradoxically does not heal well upon small damages inflicted during incision. Caution should be taken, especially upon the primary incisions, because damage will substantially complicate the suturing. The bones of very young axolots are very soft. This means that often basic anatomical forceps may suffice in bone removal. This presents another element of caution, because pinching the sinus processes could inflict substantial damage. The subcutaneous and muscle fascia layers are not available for suturing, due to their fragile tissue compositions. It is imperative to ensure a calm postoperative week. The animals may not rest sufficiently after the operation. Hence, they may inflict secondary damage to their spinal cord postoperatively. Their small anatomy does not allow for neither internal nor splice fixation.

Weight and falling height of the falling rod system is crucial to inflicting a contusion injury. During extensive piloting for an earlier study, the rod weight and falling height needed was found to be 25 g and 3 cm. This was enough to induce paralysis in 12 g axolotls without cutting or disintegrating the spinal cord. Added weight or falling height might be needed in bigger animals. Furthermore, the diameter of the falling rod might need to be bigger in the case of bigger animals and shorter for smaller animals.

The model has some limitations. Because axolots are not used for learned behavior studies, one cannot test complex neurological functions. The injury was introduced caudal to the limbs, sparing the hind limbs and bowel and bladder from being paralyzed. The reason for this was ethical, to reduce the impact on the animal to a minimum. However, it does limit the opportunity to study the effects on limb movements, which may be easier to describe and categorize. A large part of the SCI-associated morbidity stems from the loss of control of bowel and bladder. This model does not allow for future research in these fields. Inflicting damage rostral to the hind limbs would be possible, but it was not attempted.

Studying SCI in a regenerative model such as the axolotl allows for a different approach in SCI research. Because the animal model can regenerate, elimination studies will be able to reveal critical factors of regeneration. Conventional studies on SCI are performed in non-regenerative models, meaning that one will need to intervene on all critical factors to induce a regenerative response.

This model and protocol are in concordance with Krogh's principle stating that: “For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied”17. Mammalian regeneration is inhibited by multiple factors. Inhibiting these in a mammalian model usually does not induce any effects. However, increasing levels of inhibitors in the axolotl should eliminate regeneration, and thereby reveal whether that inhibitor is critical or not19.

Disclosures

The authors have nothing to disclose.

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