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A Minimally Invasive, Fast Spinal Cord Lateral Hemisection Technique for Modeling Open Spinal Cord Injuries in Rats

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TITLE:

A Minimally Invasive, Fast Spinal Cord Lateral Hemisection Technique for Modeling Open Spinal Cord Injuries in Rats

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SUMMARY:

Here, we describe a new, fast technique modeling open spinal cord injury in rats that eliminates laminectomy. Lateral hemisection is performed while viewing through a microscope. The technique is versatile and can also be used in the cervical, thoracic, and lumbar regions of the spinal cord of other animals.

ABSTRACT:

Open spinal cord injury techniques modeling laceration-like injuries are time-consuming and invasive because they involve laminectomy. This new technique eliminates laminectomy by removing two spinous processes and lifting, then tilting the caudal vertebral arch. The surgical area opens up without the need for laminectomy. Lateral hemisection is then performed with direct visible control under a microscope. The trauma is minimized, requiring only a small bone wound.

This technique has several advantages: it is faster and, therefore, less of a burden for the animal,

and the bone wound is smaller. Because the laminectomy is eliminated, there is less chance for unwanted injury to the spinal cord, and there are no bone splinters that can cause problems (bone splinters embedded in the spinal cord can cause swelling and secondary damage). The vertebral canal remains intact. The main limitation is that the hemisection can only be performed in the intervertebral spaces.

The results show that this technique can be performed much faster than the traditional surgical approach, using laminectomy (11 min vs. 35 min). This technique can be useful for researchers working with animal models of open spinal cord injury as it is widely adaptable and does not require any additional specialized instrumentation.

INTRODUCTION:

Spinal cord injuries (SCIs) are unfortunately prevalent injuries in humans. SCIs can be complicated in different ways, for example, by infections, and it is clinically important to study these injuries[1]. Because there is no single, definite cure for SCIs, animal models are still needed to further the understanding of researchers and advance possible treatments[2,3]. Although closed injuries are most commonly modeled (compression and contusion), it is clinically important to understand lacerations, which can only be modeled in open injuries[4]. Open-wound models using transection or hemisection can be used to demonstrate a more precise localization of a wound compared to closed injury models, owing to the nature of the injury (contusion vs. surgical cut). Open-wound experiments can shed light on more specific neuronal injuries in a controlled, reliable, and replicable way[5]. The complete or partial transection of the spinal cord is a widely used open-wound technique and can be viewed in detail in[6].

When studying open spinal cord injury in rats, several animals presented problems that arose from the surgery: bone splinters from the laminectomy became embedded in the spinal cord and caused swelling; the larger bone wound needed a long time to heal; the surgery took too long. An alternate surgical technique was developed to eliminate these problems. The goal was to develop a faster technique that is gentler for the animal. This newly developed technique is much faster than traditional SCI techniques. The surgical approach is minimally invasive, resulting in a smaller bone wound while eliminating problems arising from the laminectomy.

All open-wound techniques involve opening the dura[7]. Several recent studies have examined different, newly developed techniques, aiming to improve the previous methods[8,9]. Even though the opening of the dura cannot be excluded using this new technique, it causes a smaller wound on the dura while offering a reliable, controlled injury of the spinal cord. Consulting the literature on spinal cord injury techniques, many authors tried to minimize the time of surgery by implementing minor changes to the original technique[10]. Laminectomy is always part of these surgical procedures, although it is time-consuming and requires a larger bone wound to be made[6]. This surgical technique can be appropriate for researchers using open wound spinal cord injury models, specifically complete transection or lateral hemisection performed in the intervertebral spaces (**Figure 1**).

PROTOCOL:

All animal procedures were carried out according to the EU Directive (2010/63/EU) and were approved by the animal ethics committee of the Hungarian National Food Chain Safety Office (PEI/001/2894-11/2014). All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this study.

1. Preparation before surgery

1.1. Sterilize all instruments used during the procedure (see the **Table of Materials**) and disinfect the surfaces where the work is to be performed prior to the procedure.

1.2. Inject a single dose of subcutaneous antibiotics prophylactically.

1.3. Leave the animals in the operating room for 1 h to acclimate them and decrease their stress prior to surgery.

1.4. Anesthetize the rat via an intramuscular injection of a combination of ketamine and xylazine (ketamine 80 mg/kg body weight (bw) and xylazine 8 mg/kg bw).

1.5. Keep the rat warm during the procedure using a heated table or infrared light and keep the eyes moist throughout the anesthesia using physiological saline solution drops (reapply as necessary).

1.6. Fixate the animal on the operating table using surgical tape on its front and back paws and tail, and depending on the site of injury, on the neck as well. Place the rat in a stereotaxic frame to stabilize it during surgery.

1.7. Using sterile surgical suture, place a loop around the upper front teeth of the rat and fixate this on the edge of the operating table.

1.8. Pull out the tongue sideways for airway management.

1.9. Shave the fur on the back, at least 2 cm in each direction of where the incision will be made.

1.10. Disinfect the skin of the surgical area at least three times, using a povidone-iodine solution and sterile gauze. Take special care to soak the fur surrounding the area.

1.11. Assess the adequacy of the anesthesia before placing the first incision by pinching the toes and the tail of the animal. Continue monitoring the adequacy of anesthesia during the entire procedure.

2. Surgery

2.1. Place the skin incision using a scalpel blade 20. To open the surgical area, place a 2–2.5 cm long incision along the spine, cutting through all the layers of the skin. Position this incision parallel to the spinal column using the L1 vertebra as a midpoint, making it extend ~1 cm in both the cranial and caudal direction along the spinal column.

2.2. Mobilize the sides of the wound by cutting through the connective tissue surrounding the muscles.

2.3. Place two parallel incisions along the spine, penetrating the periosteum. Place the incisions right next to the spinal processes on both sides, spanning the distance between the Th13 and L1 vertebrae.

2.4. Dissect the muscles attached to the vertebrae with the aid of a raspatorium until all the spinal ligaments are visible. Put in place a retractor.

2.5. Remove the spinal processes of the 13th thoracic vertebra and the 1st lumbar vertebra using dental bone forceps to visualize the entire surgical area. Hereon, control the procedure by viewing an enlarged (4x–16x magnification) microscopic image.

2.6. Use sterile gauze to control bleeding throughout the procedure, when necessary.

2.7. Carefully lift the remainder of the L1 spinal processes, elevating the L1 vertebral arch. Sever the ligamentum flavum to access the spinal cord. Raise the caudal spinal processes further, allowing access to the spinal dura mater, which is also severed. Tip the caudal spinal processes in the cranial direction to visualize the pia mater.

2.8. Look through the pia mater for the posterior median vein, showcasing the midline of the spine.

2.9. Using the vein as a directional bisector, place an incision using a microsurgical scalpel while sparing the vein. Place the incision under the vein, in the transversal plane through the anteroposterior diameter of the spinal cord. Sever half of the spinal cord by moving the blade laterally away from the centerline.

NOTE: The incision is unilateral, on the right side, at the 4th lumbar segment.

2.10. Try to place the incision to avoid cutting the anterior spinal artery. Ensure excess pressure is not applied on the vertebral body when cutting the spinal cord to spare the anterior spinal artery on the ventral side of the spinal cord.

[Place Figure 1 here].

2.11. Do not close the dura mater directly during wound closure. Tightly suture (suture size 4-0) the muscles along the spinal processes, indirectly closing the small wound on the dura mater.

2.12. Close the dorsal connective tissue layer with sutures.

2.13. Finally, suture the skin around the incision site.

3. Postsurgical care and follow-up

3.1. Allow the animals to awaken in their cages. Keep the animal(s) warm using a heat lamp in addition to the temperature-controlled room. Do not leave the rats alone after they awaken after surgery, and do not put them together with other rats in the same cage.

3.2. Monitor their respiratory rate at least every 10 min until they are fully awake. If necessary, apply gentle stimulation (e.g., rub the head) to aid their awakening from anesthesia.

3.3. When the animals are alert and appropriately active, transport them safely back to the animal house.

3.4. Keep the rats under close surveillance for the first 24 h post surgery. Following the first 24 h post surgery, check the animals at least twice a day until the end of the experiment, monitoring for signs of distress.

3.5. Thoroughly assess them once a day for signs of distress using the relevant institutional animal welfare protocol and take special care to check their wounds for signs of infection and inflammation.

NOTE: Stress and infection affect the welfare of the animals and the outcome of experiments.

3.6. Administer antibiotics intramuscularly every day until the end of the experiments. Keep the animals in sterile cages, one animal per cage, and give food and water *ad libitum*, same as before. At the end of the experiments (or if any serious adverse reaction is observed during the timeframe of the experiment), euthanize the animals humanely, in accordance with the relevant institutional animal welfare protocol.

NOTE: Here, the animals were euthanized (in a deep sleep induced by the combination of ketamine–xylazine) by first administering a physiological saline perfusion (1/3 mL/g bw) followed by a 4% paraformaldehyde perfusion (1 mL/g bw).

REPRESENTATIVE RESULTS:

Following the hemisection, the rats show paralysis in the ipsilateral hindlimb (*in vivo* proof of successful hemisection). Thorough specimen evaluation can only be done following the removal of the spinal cord (see **Figure 2**, where the removed spinal cord can be seen from both the ventral and dorsal sides).

[Place Figure 2 here]

First, the removed spinal cord is analyzed in its entirety under a microscope using 4x–16x magnification (to evaluate the degree and precision of the injury). The specimen is then further analyzed using histology, where the site of the injury can be seen in greater detail. Hematoxylin and eosin (H&E) stain was used to prepare the samples (**Figure 3**).

[Place Figure 3 here]

Figure 2 and **Figure 3** show that the incision is perfectly acceptable in length and placement. The quality of the samples was at least as good as those obtained from animals whose spinal cords were hemisected using the traditional surgical approach with laminectomy (for a detailed description of the traditional surgical method, see [6]). The images are not qualitatively different from the result of any other surgical approach, even though this technique is faster and there is no laminectomy.

The results show that this technique can be performed much faster than the traditional surgical approach using laminectomy (11 min vs. 35 min). The spinal cord is exposed for 10–15 s with this method, compared to a minimum of 3.5 min using laminectomy (until the closure of the dura). In conclusion, this new minimally invasive SCI method without laminectomy is much faster and does not require any additional specialized instrumentation.

FIGURE AND TABLE LEGENDS:

Figure 1: Artwork showing the steps of the new open SCI technique in rats. (**A**) The exposed vertebrae. (**B**) Spinal processes removed (Th13 and L1). (**C**) The lifted and tilted vertebral arch of the L1 vertebra. (**D**) Hemisection performed on the right side, with hemisected spinal cord shown separately, zoomed in.

Figure 2: Ventral and dorsal views of the removed spinal cord following hemisection. The entire removed spinal cord viewed from the ventral side (**A**) and the dorsal side (**B**) shown side-by-side.

Figure 3: Histological sample showing hemisection. Histological sample stained using hematoxylin and eosin, showing the hemisection, viewed under a microscope (16x magnification). Scale bar = 1 mm.

DISCUSSION:

This minimally invasive spinal cord injury technique was developed when studying spinal cord-injured rats, and the team was faced with problems arising from the surgery itself (bone splinters from the laminectomy causing compression and damaging the spinal cord, surgery taking too long, slow healing of a large bone wound). By eliminating the laminectomy, the procedure became much faster (11 min vs. 35 min), the structure of the vertebral canal remained intact, the bone wound was much smaller, and there were no bone splinters that could damage the spinal cord.

The removal of the spinous processes cannot be eliminated because the removal of the upper (cranial) spinous process is necessary to tilt the lower (caudal) spinous process backward. The removal of the lower spinous process greatly improves the visibility of the spinal cord, facilitating hemisection.

The hemisection is the most critical part of the protocol. Here, the hemisection is performed freehand, although this is not a prerequisite. A stereotaxic instrument can be used instead. The rat can also be placed in a stereotaxic frame to stabilize the animal during surgery[6]. This step will require only a slight modification in the technique outlined here. This can also be helpful if someone with little experience is performing the procedure.

This new technique is extremely versatile. Here, the procedure was performed at the L4 lumbar segment (L1 vertebra); however, it can be used in other segments of the spinal cord tailored to the specific needs of the actual experiment (this technique has been used in the thoracic and the cervical regions as well). It could also be easily adjusted to implement a complete transection of the spinal cord instead of a hemisection. Lifting the vertebral arch allows direct inspection of the given part of the spinal cord. Thus, a small disc of spinal cord tissue can also be removed to ensure complete transection.

The use of this new technique is not limited to rats but can also be applied to other species used to model spinal cord injuries (e.g., mice, pigs, dogs). The main limitation of this technique is that because the hemisection (or transection) can only be performed in intervertebral spaces, it is not suitable for those who specifically need the cut to be placed in the vertebral spaces. Moreover, because it is an open-wound technique, it is not optimal for modeling contusions or compression injuries.

However, this technique can be the ideal choice for studying open SCI, as the hemisection (or transection) is executed precisely and is easily reproducible. Spinal pathways can also be studied with fewer artifacts as the vertebral canal remains intact. It can be especially useful when studying minimally invasive therapeutic approaches. Using this technique, the focus of attention can be solely on the treatment instead of on possible side effects of the surgery[11].

In conclusion, this new, minimally invasive technique requires neither new equipment nor expensive settings as only equipment readily available in laboratories working with animals is utilized. It can easily be adapted to the specific needs of a given study (site of injury; hemi- or transection; type of animal). It is also easy to learn. Therefore, this modification could be of interest to researchers working with open SCI animal models.

ACKNOWLEDGMENTS:
The authors wish to thank Gergely Ángyán for the original artwork. This research work was funded by Semmelweis University, Budapest, Hungary. This study was also supported by the Hungarian Human Resources Development Operational Program (EFOP-3.6.2-16-2017-00006). Additional support was received from the Thematic Excellence Programme (2020-4.1.1.-TKP2020) of the Ministry for Innovation and Technology in Hungary, within the framework of the Therapy thematic program of Semmelweis University.

DISCLOSURES:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1.: Artwork showing the steps of new open SCI technique in rats.

Click here to access/download;Figure;Fig1.jpg

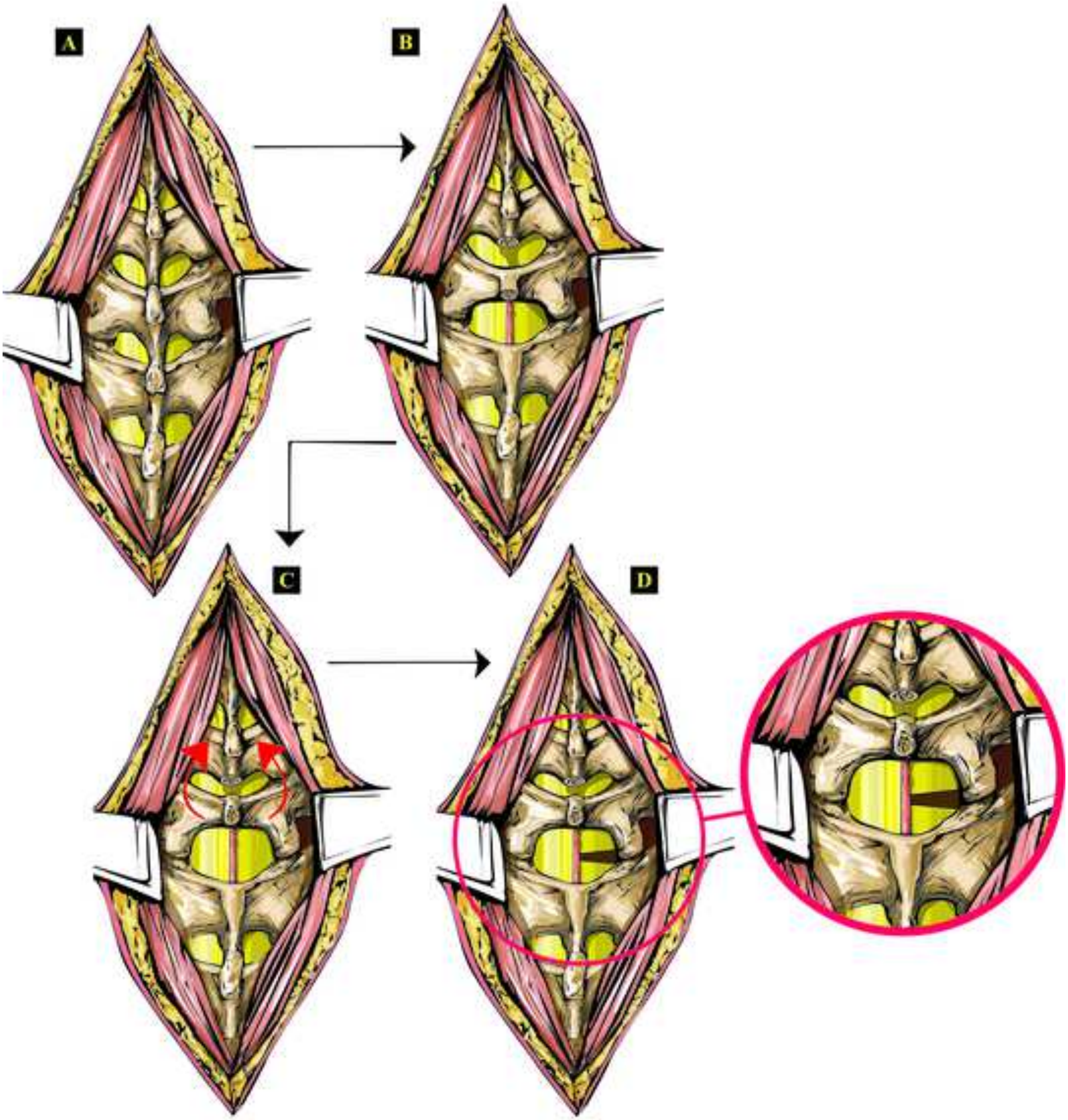

Figure 2.: Ventral and dorsal view of the removed spinal cord following hemisection.

Figure 3.: Histological sample showing hemisection.

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Table of Materials
JoVE_Materials 2 (1).xls

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Manuscript revised for spelling and grammar.
2. Please revise the title to more clearly define the model system used and the type of study design. Please avoid the use of punctuation in title – Title revised.
3. Please do not use bullets in the summary. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words. Summary rewritten: "New, fast technique modeling open spinal cord injury in rats, eliminating laminectomy. Lateral hemisection is performed under direct visible control through microscope. The technique is versatile, since it can be used in the cervical, thoracic and lumbal region of the spinal cord and its use is not limited to rats."
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Text revised and rephrased throughout to eliminate any personal pronouns.
5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) and before the punctuation. Revised.
6. Please also include in the Introduction the following with citations: Revised: Introduction was revised to include the following points.

a) A clear statement of the overall goal of this method – The goal was to develop a faster technique that is gentler for the animal.

b) The rationale behind the development and/or use of this technique - when studying open spinal cord injury in rats several animals presented problems that arose from the surgery itself (bone splinters from the laminectomy became embedded in the spinal cord and caused swelling, the larger bone wound needed a long time to heal, the surgery itself took too long). To eliminate these problems an alternate surgical technique was developed.

c) The advantages over alternative techniques with applicable references to previous studies – faster surgery, smaller bone wound necessary.

d) A description of the context of the technique in the wider body of literature – aiding in modeling open-wound spinal cord injuries.

e) Information to help readers to determine whether the method is appropriate for their application - using open wound spinal cord injury models, specifically complete transection or lateral hemisection that is performed in the intervertebral spaces.

7. Line 78: Please provide the concentration of anesthesia used. Concentration provided.
8. Line 122: Suture size? Revised.
9. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Protocol revised.
10. Please include a single line space between each step, substep, and note in the protocol section. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Protocol revised and highlighted.
11. Representative results: How many rats underwent this procedure? Counting

altogether, in several different experiments, this surgical procedure has now been performed on more than 100 rats.
12. Line 143: How was paralysis assessed? Was it a visual quantification or was any other method used? Paralysis of the ipsilateral hindlimb was confirmed visually.
13. Line 145: How was the spinal cord removed? Please describe in brief. Following euthanasia (according to protocol), a formalin perfusion was administered. The vertebral column was removed. All the vertebrae were then cut up (using scissors), on the abdominal side on both sides of the vertebral body. The spinal cord was then removed for further preparation for histology.
14. Lines 150 to 155: please provide references to previous studies which were used to compare the results. Was any statistical analysis done? If yes, please provide the details. Revised. Reference provided. No statistical analysis was done.
15. Figure 2,3,4: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend. Images and figure legends revised. Scale bar included.
16. As we are a methods journal, please also include in the Discussion the following in detail along with citations: Discussion revised.
a) Critical steps within the protocol – revised: the hemisection is the most critical part of the protocol.
b) Any modifications and troubleshooting of the technique – revised: stereotaxic instrument can be used.
c) Any limitations of the technique – limitations – revised: intervertebral spaces only, open-wound technique only.
17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. References revised.
18. Please ensure that the table of materials contains information regarding all the essential supplies, reagents, and equipment used in the study. Table of materials revised.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Csomo and colleagues suggested a new method of carrying out a lateral hemisection without the need to removal of the vertebra so reducing the surgery time. Anything that can reduce surgery time without compromising the animal or surgery is always a good strategy.
The general strategy of the surgical approach seems logical and fine. However, there are issues with the manuscript in its current form.

Major Concerns:
1-Hemisection can be either be dorsal or lateral, so need to state 'lateral hemisection' in the title. Title revised.
2- Line 40. Statement 'our technique can be useful for others working with animal models of spinal cord injury' suggest this procedure can be carried out anywhere along the spinal cord. If the authors suggest this is incorrect, then they would be narrow their statement. If it is correct, then this needs to be mentioned in the

discussion that this is possible and how. Discussion revised: This new technique is very versatile. Here the procedure was performed at the L4 lumbar segment (L1 vertebra), but it can be used in other segments of the spinal cord tailored to the specific needs of the actual experiment (this technique has been used in the thoracic and the cervical regions as well).
3- line 119-120. 'make sure you do not apply too much pressure on the vertebral body'. This is important part to get right as too little pressure will cause incomplete transection and too much will cause damage to anterior spinal artery. Therefore, apart from the applied pressure, is there any other information to enable the reader to reproduce this model successfully without practicing on many animals? Discussion revised. Stereotaxic frame can be used for the less experienced operator and to stabilize the animal.
4-Why was analgesic missing in the protocol? Was it not used? Ketamine-xylazine combination was used for anesthesia, which produces short-term surgical anesthesia with good analgesia. It does not require additional analgesia. Please see for example the guidelines for rodent anesthesia and analgesia of the University of British Columbia (Rodent Anesthesia and Analgesia Formulary and General Drug Information (2016) (Guideline))
5-line 135. Author mentioned about 'monitoring for signs of distress'. How often do you expect distress to occur using this model? What is the procedure if the animal showed signs of distress? When working with animals it is always a top priority to care for their welfare, which is why it is emphasized in the protocol to always monitor for distress. Adverse event could be that the anesthesia wears off, though this is very rare and needs to be addressed immediately. Every laboratory working with animals must adhere to strict protocols concerning animal welfare and have safeguards in place to ensure the least amount of suffering.
6-It would be more convincing if there are multiple images of the lateral hemisection at dorsal and ventral view of different animals displayed side by side as it will show the reproducibility of the surgical technique. We believe the video will be instructive but multiple images is a good idea as well.

Minor Concerns:
1-line 5: 'unwanted injury' may be a better term. Revised.
2-line 51. Statement 'closed injury models are not as precise as open-wound models' requires a reference. Revised: Open-wound models using transection or hemisection can be used to demonstrate a more precise localization of a wound compared to closed injury models, owning to the nature of the injury (contusion vs. surgical cut).
3-line 55. Unclear what 'major artefacts' are if any. Revised.
4-line 97 and 112. Unclear the term 'capitally'. Should it not be cranially' or 'rostrally'? Revised, exchanged for cranially.
5-line 101. Unclear how the periosteum can be 'penetrated' since it's below the vertebra surrounding the spinal cord. The periosteum is the membrane covering the outer surface of all bones.
6-line 113: posterior median vein Revised.
7-line 139-140. Unsure the term 'sterile cage'. Was it really sterilised and how often was the cage changed when soiled? Also, potential welfare issue if one animal per cage continued throughout the study, so why need to keep the animal individually housed. The cages are sterilized before the animal is placed in it following the surgery. The animals are closely monitored for the first 24 hours after surgery and are then checked for distress (according to the animal welfare protocol of the

institute) and tended to (this includes cleaning the cages and providing food and water) twice a day. The reason for keeping the animals apart is so they will not pick on each other's wounds (and so they will not harm each other which is common when there is a wound).

Reviewer #2:
Manuscript Summary:
The manuscript sufficiently describes the procedure.

Major Concerns:
The utility of the procedure needs to be described better. As described, this is a hemisection injury but it is compared to compression or contusion injury. Revised to make the description clearer. This technique is not compared to compression or contusion injuries; these are simply stated as being the most common type of injuries modeled. However, open-wound injury models are still needed as they offer a chance to model more precise injuries, like lacerations as well as in situations where the precise location of the injury is important (for example when studying neuronal regeneration, please see Borbély et al., 2017). The injury model described here cannot replace a compression or contusion injury as they are designed to study different aspects of regeneration. This technique aims to improve existing open-wound techniques and is not meant to replace compression or contusion injuries.
In addition, the authors state that a "traditional surgical approach using laminectomy" takes 35 minutes but they never describe what a constitutes a "traditional surgical approach using a laminectomy." Revised. Traditional surgical technique is referenced several times. This is important because currently there are several injury models that use laminectomy in rats. Furthermore, no papers are referenced as to what are the side-effects of the length of the current procedures. Working with animals it is important to adhere to the 3Rs principle (replace, reduce, refine). This new technique is less invasive and at the same time faster which is a refinement of the existing traditional technique.

The authors also states that this surgical approach can be done in different parts of the spinal cord as well as in other species. No evidence of this is shown, and no data is presented as to the length of such procedures. Revised the discussion.

One of the advantages of performing a laminectomy is that it mimics the removal of vertebrae in order to reduce the swelling of the spinal cord after the primary injury (compression, contusion, or hemisection). In this model, the swelling of the cord presents a source of continues damage since it will press against the vertebrae. This work does not present any behavioral data to show that the function of the animal does NOT deteriorate as a consequence the spinal cord swelling after the primary injury. Bone splinters (from the laminectomy) can become embedded in the spinal cord causing swelling (please see Ashammakhi, N. et al., 2019) which was one of the problems we tried to eliminate by developing this new technique. The spinal cord itself can also be damaged when the laminectomy is performed. Laminectomy results in a much larger bone wound than what is necessary with this technique. The spinal canal remains intact and offers protection. Working with this technique, the focus of the experiment can be on whatever treatment is being researched instead of on the possible side-effects of the surgery. In the scope of this article, we focused on the new method we developed, and the aim was to show this method so others can use it too. Behavioral data is of course a very good suggestion and could be a new project.