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Intracavernous Pressure Recording in a Cavernous Nerve Injury Rat Model.

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TITLE:

Intracavernous Pressure Recording in a Cavernous Nerve Injury Rat Model

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SUMMARY:

This protocol describes developing a stable bilateral cavernous nerve injury rat model of radical prostatectomy associated with erectile dysfunction and intracavernous pressure measurement.

ABSTRACT:

The bilateral cavernous nerve (CN) injury rat model has been extensively used to simulate clinical cavernous nerve injury associated with erectile dysfunction (ED) for evaluating the effect of clinical therapeutic methods. However, the methods of CN injury model construction are flawed and varied in the ED research field. It is CN crush injury that is the most commonly used method in recent years. This study aims to provide a detailed description of the procedure of bilateral CN injury rat model construction and measurement of intracavernous pressure (ICP) recording, providing a reliable and reproducible CN injury rat model. This work successfully developed the CN injury method of hemostat crush injury using a syringe needle as hard support and a hemostat with a rubber sleeve. Also, this method concludes that a voltage of 1.0 V, frequency of 20 Hz, and pulse-width of 5 ms are the optimized stimulation parameters for ICP recording in a bilateral CN injury rat model.

INTRODUCTION:

ED is one of the common diseases in adult men. It is estimated that the number of ED patients in the world will reach 322 million by 2025[1]. One multicenter extensive sample survey in China shows that the proportion of ED caused by pelvic surgery or trauma is about 8%[2]. Despite the continuous improvement of surgical techniques and surgical instruments, the incidence of ED is still high. It has been considered that the development and progression of ED after nerve-sparing radical prostatectomy (RP) contributes to cavernous nerve injury resulting in atrophy of corpus cavernosum smooth muscle, apoptosis of endothelial cells, and pathological remodeling[3,4].

For studying the mechanism of hemodynamics and histopathology changes of CN injury associated with ED, several different types of CN injury animal models have been developed and assessed, including rodent, dog, cat, and monkey[5-7]. Relying on the advantages in expenditure and reproducibility, the bilateral CN injury rat model has become the most common model for assessing ED after radical pelvic surgery[8]. However, various forms of nerve injury have been reported in numerous literature whose principal differences are nerve injury approaches (crush, freezing, transection, and excision)[9-11]. Furthermore, the diversity of nerve injury approaches might lead to inconsistency in intracavernous pressure (ICP) recording parameters in the rat model, which determines the accuracy and evaluation of ICP[8]. Nevertheless, there is not a standardized method for inducing nerve injury and recording ICP of the model yet.

Therefore, this study aims to build a more reliable and reproducible bilateral CN injury rat model. This method provides a detailed description of the procedure of model construction and ICP measurement, which might be beneficial to study the mechanisms of ED and develop effective treatments in the future.

PROTOCOL:

Fifteen adult male Sprague–Dawley rats (3-month-old) weighing between 300-350 g were used in this study. All animal procedures were performed following the NIH Guidelines for the Care and Use of Laboratory Animals and with the approval of The fifth affiliated hospital of Sun Yat-Sen University Institutional Animal Care and Use Committee. Animals were housed in a comfortable facility with temperature and light controlled.

1. Preparation for surgical procedure materials

1.1. Prepare the following instruments: scalpel, tissue scissors, thread scissors, bending forceps, tissue forceps, microsurgery forceps, Hartman mosquito hemostatic forceps, sterile surgical sheets, a microneedle holder, rat abdominal retractors, and biological signal acquisition and processing system (see **Table of Materials**).

1.1.1. Sterilize all surgical instruments before operation. Use alcohol (70% ethanol) wipes to clean the surgical area.

NOTE: The surgical instruments should be sterilized by alcohol immersion overnight.

1.2. Prepare the pressure recording system

1.2.1. Connect a 10 mL syringe containing heparin saline and a hypodermic 25 G needle to a 3-way stopcock with a tube (20 cm length). Flush the sterilized tube with sterile heparin saline (200 U/mL).

NOTE: Filling the tube with heparin saline avoids introducing air bubbles into the system.

1.3. Lift the 25 G needle 20 cm (just the tube length) above the animal operating pad. Then examine the measurement accuracy of the pressure recording system by flushing or tapping.

2. Preparation of the animal

2.1. Anesthetize rats by sodium pentobarbital (60 mg/kg) intraperitoneal injection (see **Table of Materials**).

NOTE: To confirm sufficient depth of anesthesia, an evaluation of spontaneous breathing rhythm and the reflexes of a rat *via* pinching hind paw was performed.

2.2. Apply ointment on bilateral eyes to avoid corneal dryness.

2.3. After confirming a proper anesthetization, shave the lower half of the abdomen, neck, and perineum using an electric shaver. Place the rat in the supine position on a heating pad (37 °C). Wear medical gloves to maintain sterile conditions during surgical procedures.

3. CN isolation and injury procedure

3.1. Use a scalpel to make a 4 cm incision through the skin at the lower, midline abdominal. To fully expose the bladder and the prostate, use tissue scissors and tissue forceps to make a proper length incision through the subcutaneous fascia, the muscular tissue, and the peritoneum.

3.2. Use a rat abdominal retractor to enlarge the operative field map. Use absorbent cotton swabs to separate the prostate from the adjacent tissues, such as ligaments.

NOTE: Major pelvic ganglion (MPG) and CN could be found at one of two the dorsolateral areas of the prostate.

3.3. Use angled micro scissors to incise the fascia overlying CN 1-6 mm distal to MPG. Then slide a 9-0 suture under the CN with the use of microsurgery forceps.

3.4. Place a syringe needle (25 G) underneath the CN, 5 mm distal to MPG. Then put the hemostat in the light of the "hemostat tip-syringe needle-nerve-hemostat tip" sandwich structure (**Figure 1** and **Figure 2**).

NOTE: The syringe needle needs to be ground flat.

3.5. Apply the hemostat with full tip closure at 5 mm distal from the ganglion for 1 min, then withdraw the hemostat and the syringe needle (**Figure 2**).

3.6. Uplift the nerve slightly *via* a 9-0 suture, and place the hooks of the bipolar electrode (see **Table of Materials**) around the CN 2-4 mm distal to MPG (**Figure 3**).

NOTE: Two pairs of MPG and CN were operated in the same way.

4. Catheterization of the corpus cavernosum and stimulation of the CN for ICP measurement

4.1. Flush the tube with sterile heparin saline (200 U/mL) before introducing it into the corpus cavernosum.

4.2. Hold the 25 G needle and keep the insert direction parallel with the course of the corpus cavernosum (**Figure 3**).

NOTE: The tunica albuginea should be stretched to facilitate the insertion.

4.3. Push the 25 G needle 6 mm into the corpus cavernosum (**Figure 3**). Flush the tube and press the corpus cavernosum lightly to evaluate the sensitivity of the transducer (**Figure 4**). To prevent accidental falling off, fix the pipe on the worktable with adhesive tape.

4.4. Use the following parameters for CN stimulation: voltage at 1.0 V, frequency at 20 Hz, pulse width at 5 ms. Apply 1 min of stimulation with 5 min of rest between the following stimulation.

NOTE: Turn the 3-way stopcock to the pressure transducer channel when starting the measurement.

5. Postoperative Care

5.1. Place the rats on a warmed pad (37 °C) and monitor them carefully for anesthesia recovery.

5.2. For postoperative pain control, provide non-steroidal anti-inflammatory drugs (such as Carprofen, 0.5 mg/kg, subcutaneous injection) (see **Table of Materials**) when the rats fully recover.

5.3. Move rats to the aseptic cage and monitor them 2 days to evaluate the incisional wound's nourishment state, mental state, and infection.

REPRESENTATIVE RESULTS:

The surgery procedure produced a typical ICP response curve using this protocol with the recommended stimulation settings. The ICP response curve rises instantly when stimulating the nerve and drops when the stimulation is withdrawn (**Figure 5**). It is essential to examine the intracavernous pressure line before measuring the ICP, which affects the evaluation of increased ICP values (**Figure 4**).

As illustrated in **Figure 6**, there is no significant difference between the peak ICP and plateau of ICP when voltage is above 1.0 V on normal rats (without cavernous nerve injury). However, the peak ICP and plateau of ICP increase with increasing stimulation voltage above 1.0 V after cavernous nerve injury (**Figure 7**). The ICP measurement was assessed at pre-operation, 0, 7, and 28 days following CN crush. There was a significant difference of ICP between 0 days and 7 or 28 days of post-operation, but no statistical difference between 7 days and 28 days (**Figure 8**). It indicates that the CN injury rat model following the current method is reliable.

FIGURE LEGENDS:

Figure 1: The instruments of hemostat crush injury. (**A, B**) The hemostat with a rubber sleeve. (**C-E**) The simulative structure of "hemostat tip-syringe needle-nerve-hemostat tip" is shown.

Figure 2: The procedure of cavernous nerves injury. (**A**) The anatomical structure of the MPG and CN (marked by a red line). (**B**) Placing a syringe needle underneath the CN with a certain angle (red arrow). (**C**) A hemostat was applied to CN to perform injury.

Figure 3: Catheterization of the corpus cavernosum and Hooking of the nerve. (**A**) 25 G needle was parallel with the course of the corpus cavernosum when catheterizing. (**B**) Pushing the 25 G needle into the corpus cavernosum. (**C**) Placing the nerve on the hooks of the bipolar electrode.

Figure 4: Examing the intracavernous pressure line. The sensitive response curve suggests that the 23 G needle is in the correct position of intracavernous.

Figure 5: The typical ICP response curve of normal rats. When starting stimulating CN, the ICP quickly rises and enters a plateau. The ICP decreased to baseline without stimulation.

Figure 6: The effect of voltage gradient stimulation on ICP without cavernous nerve injury. With increasing stimulation voltage above 1.0 V, the peak ICP and plateau of ICP don't increase.

Figure 7: The voltage gradient stimulation on ICP with real-time cavernous nerve injury. With increasing stimulation voltage above 1 V, the peak ICP, and plateau of ICP increase.

Figure 8: The measurement of ICP at different post-operation times. ICP decreases maintains a lower ICP level up to 28 days.

DISCUSSION:

ED is a severe complication of pelvic surgery or trauma. Although undergoing a nerve-sparing operation, the incidence rate of ED is approximately 14-90% in radical prostatectomy (RP)[12]. Due to the problematic regeneration of injury CN, the clinical curative effect is less than satisfactory. Thus, a stable CN injury animal model for exploring treatments of ED is essential. Quinlan et al. first reported the CN injury rat model for the study of RP-associated ED[13]. Several studies developed CN injury rat models based on the Quinlan model, including transection, excision, crush, and freezing of the CN[8,14-17]. Each type of injury could be performed unilaterally or bilaterally for a particular experiment design.

Despite the least severe degree of injury, crush type can reserve the perilemma epineurium of the CN. Bilateral CN crush injury is the best analogy to nerve-sparing RP[18,19]. Nevertheless, there exist some problems with the methods of CN crush injury reported in the current study. Lack of a sufficient degree of injury and multiple injuries limit the application of the model. A single-point injury model with an adequate degree has an unparalleled advantage in basic research. Therefore, we had developed a more stable bilateral CN injury rat model of RP-associated ED.

CN is liable to neurotmesis because of its' slender size. This study first proposed an operating skill to ensure adequate injury degree and avoid nerve transection, using a syringe needle as rigid support and a hemostat with a rubber sleeve. Nevertheless, different compression forces and times would determine the degree of injury that influences the success rate of model construction. The current study found that applying a hemostat with full tip closure at 5 mm distal from the ganglion for 1 min might be the most appropriate operating mode.

For evaluating the stability and reliability of the model, erectile function recovery was assessed at 0, 7, and 28 days following CN crush. It was found that there was a significant difference of ICP between 0 days and 7 or 28 days; however, there was no significant difference between the ICP values of the 7 days and 28 days. It indicates that erectile function degenerates gradually and appears to maintain a lower ICP level up to 28 days. This suggests that the Bilateral CN crush injury rat model is suitable for a one-month experiment design.

The CN stimulation voltage in studies doesn't have a general agreement, which varies from 1.0 to 12 V. Firstly, the effect of voltage gradient stimulation on the ICP was explored in normal rats. With increasing stimulation voltage above 1.0 V, the peak ICP and plateau of ICP don't rise. Our result is in accordance with Hox, M. et al.'s work[20]. This phenomenon suggests that the current conducted *via* the nerve is above the threshold and sufficient to trigger the reflex resulting in a complete physiological response. After being injured, CN was instantly stimulated by gradient voltage, and ICP was recorded. Compared with 1.0 V, the peak ICP and plateau of ICP increase with increasing stimulation voltage above 1 V. Using a higher stimulation voltage might lead to a "false positive" ICP response curve. In general, using a voltage of 1.0 V, frequency of 20 Hz, and pulse-width of 5 ms as stimulation parameters for ICP recording in a bilateral CN injury rat model is recommended.

As with other animal models, the bilateral CN injury rat model *via* the current method also has

some limitations compared with clinical patients. Rat model with the better regenerative ability of the peripheral nervous system might influence the evaluation of nerve regeneration and recovery. In contrast, it provides an acceptable research method in the current study. Therefore, it is necessary to establish a more stable bilateral CN injury rat model of ED, contributing to achievements transformation in clinical treatment.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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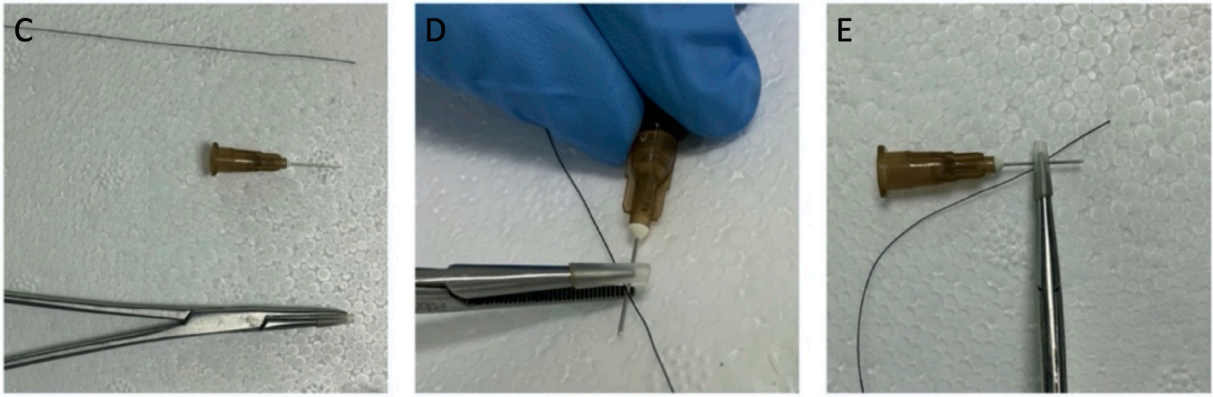

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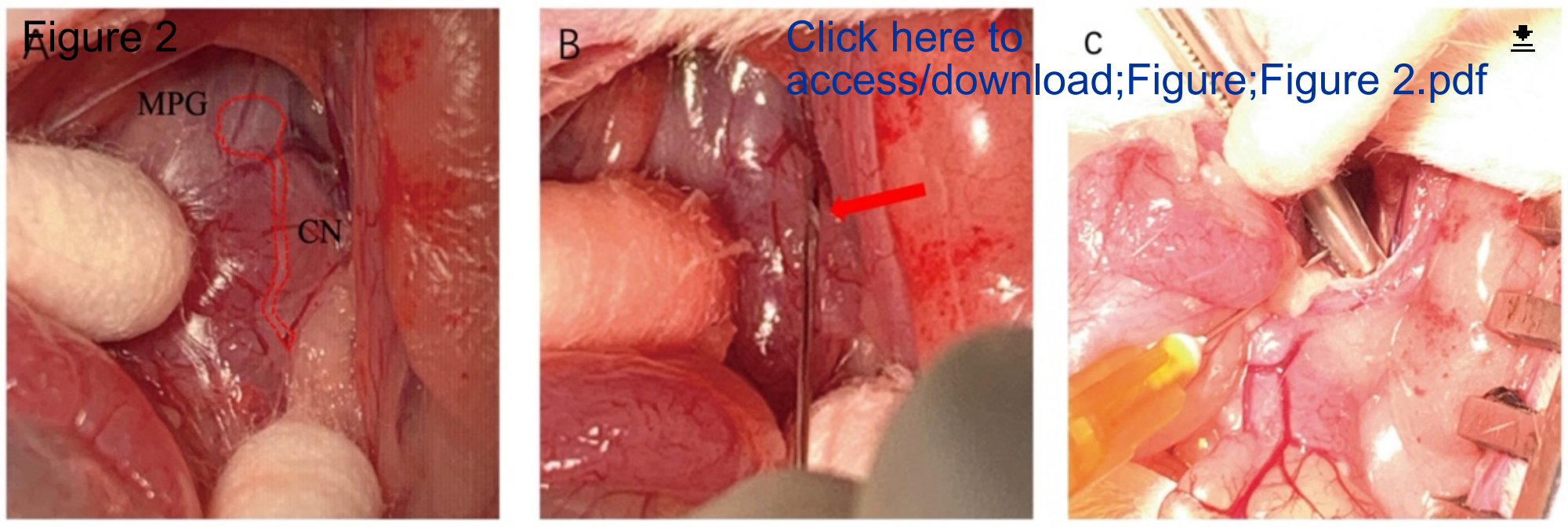

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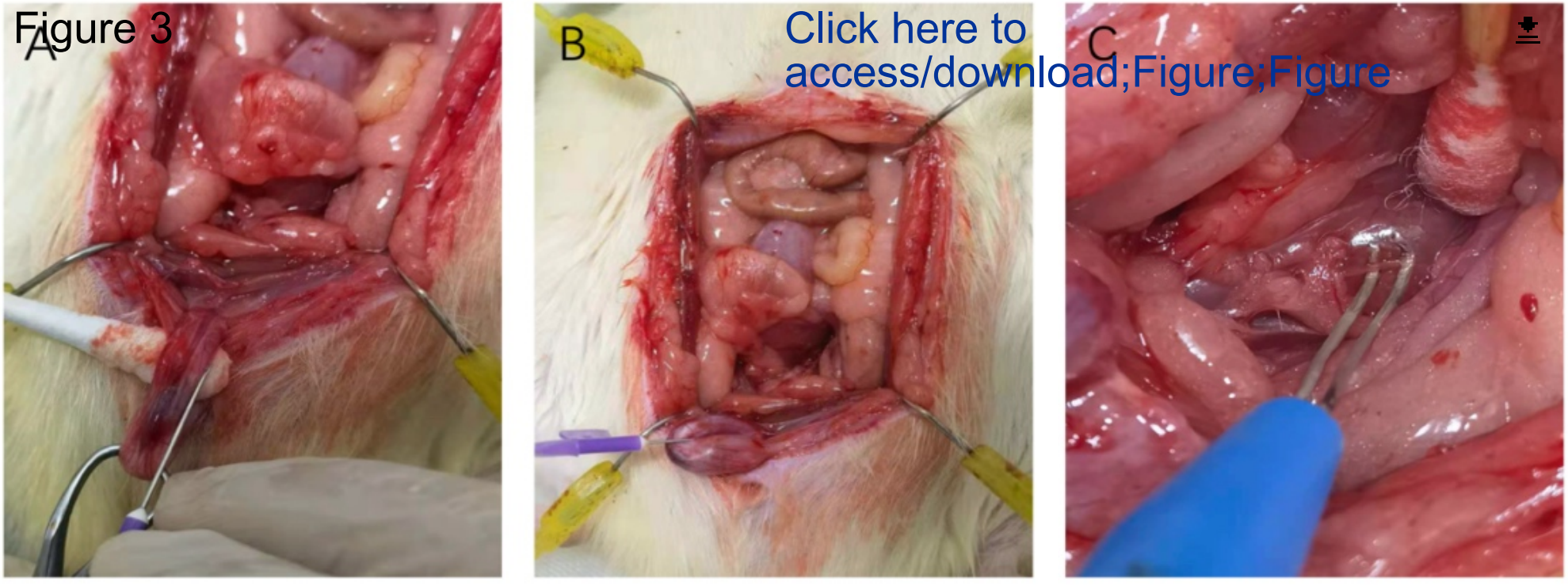

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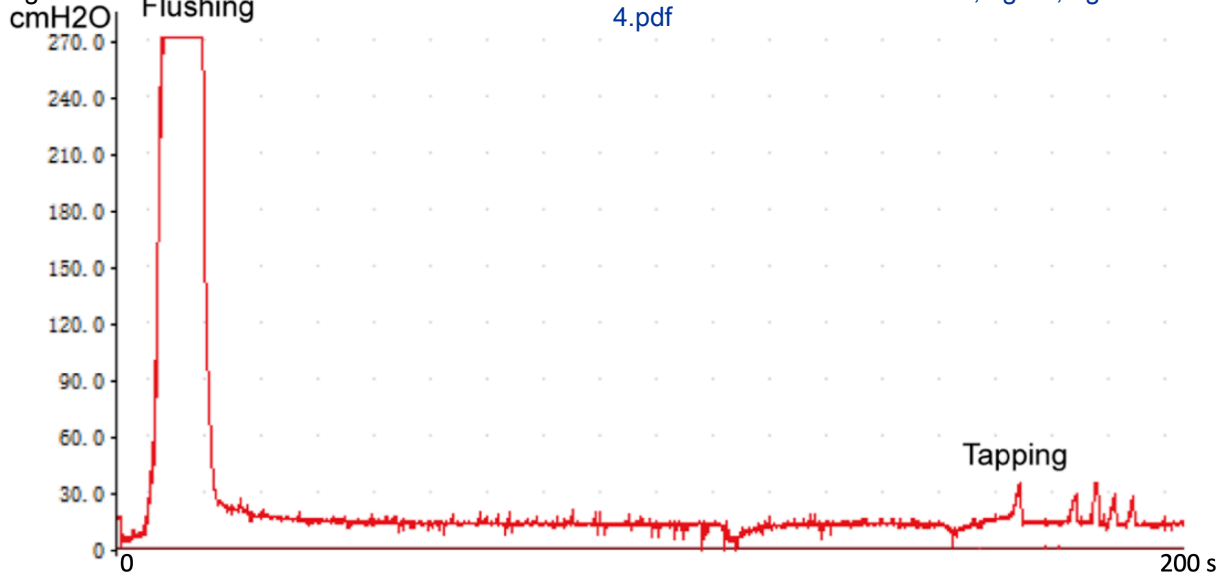

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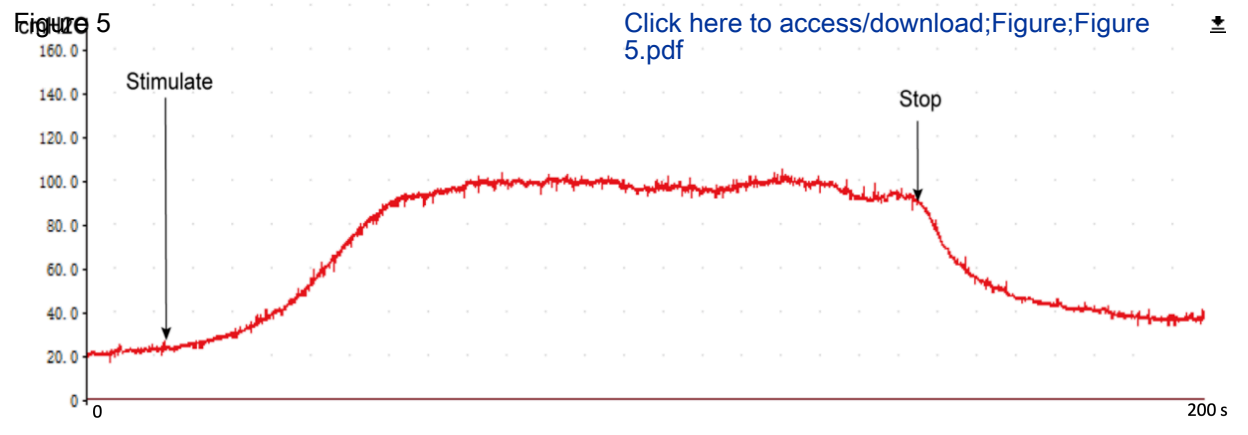

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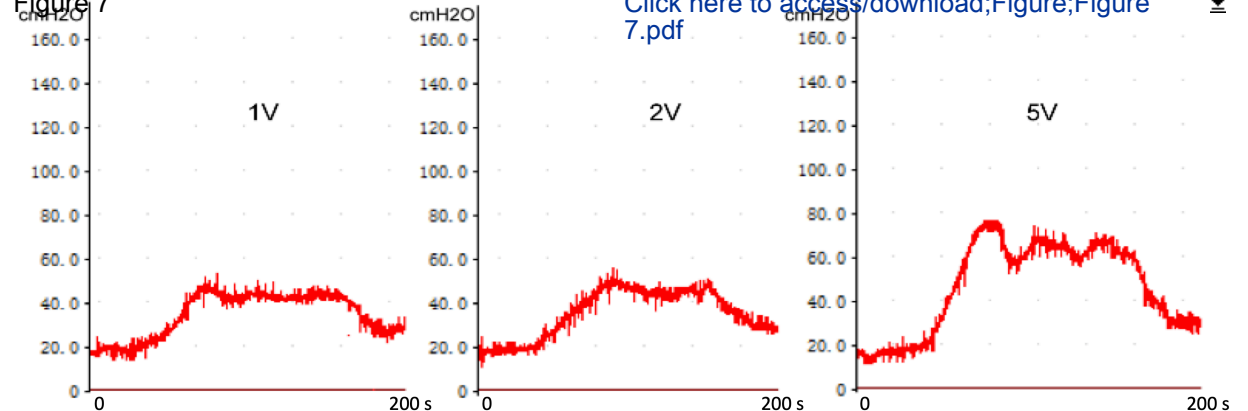

Figure 8

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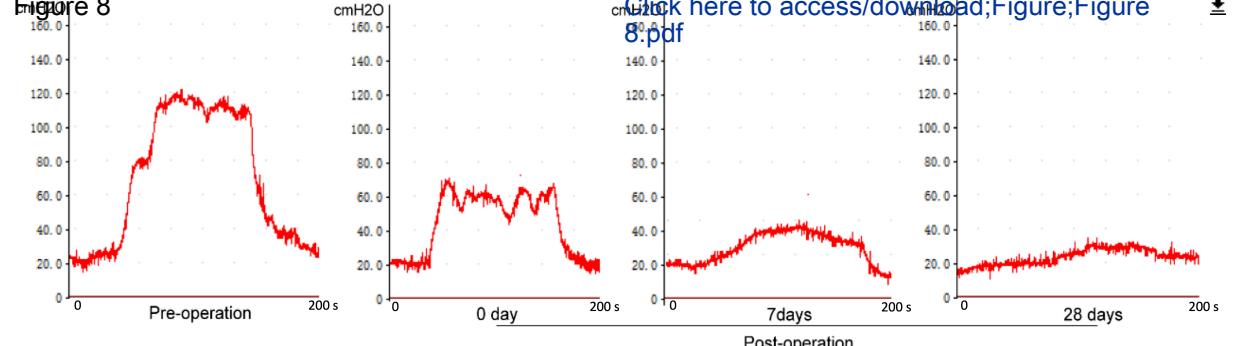

Dear Editor,

Thank you for your letter and for the comments concerning our manuscript entitled “Intracavernous Pressure Recording in a Cavernous Nerve Injury Rat Model” (ID: JoVE63024). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches.

We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in the manuscript with corrections tracked. And we have provided brief replies to each comment in the manuscript.

Thank you very much for your considering our manuscript for potential publication. I'm looking forward to hearing from you soon.

Sincerely yours,

Zitaiyu Li

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Intracavernous Pressure Recording in a Cavernous Nerve Injury Rat Model" (ID: JoVE63024). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Responds to the editorial comments:

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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. Please define all abbreviations at first use.

2. Please provide an email address for each author.

3. Please revise the following lines to avoid overlap with previously published work: 76-77, 78-79.

4. Please increase the word count of the abstract to be 150-300 words.

5. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

6. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) What happened to the rats after the study? Please specify the euthanasia method without highlighting it.

b) Please mention how proper anesthetization is confirmed.

c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

e) Discuss maintenance of sterile conditions during survival surgery.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

8. Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol.

9. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

10. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. The legends should appear only in the Figure and Table Legends section after the Representative Results.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

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author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

13. Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

Response:

1. We are very sorry for our incorrect writing. Spelling mistakes has been checked and corrected all over the text.

2. Institutional email addresses for all authors have been listed in manuscript.

3. Manuscript of lines 76-77, 78-79 has been revised.

4. The word count of the abstract has been increased within the range of 150-300 words .

5. The introduction section has been revised as required.

6. More detailed information of animal treatment has been added to protocol section of the manuscript.

7. The manuscript format has been corrected as required.

8. All figures have been discussed in the representative results, and figures showing the experimental setup have been referenced in protocol section.

9. Representative results have been explained as required.

10. All embedded figures have been removed from the manuscript. All figures have been loaded as a figure file.

11. The discussion section has been revised as required.

12. The references format has been corrected for proper format.

13. The Table of Materials has been add all items and sorted by the name of the material.

Special thanks to you for your good comments.

Reviewer #1:

Response to comment:

1. Although it described how to catheterize the corpus cavernosum, I suggest to add some detail figures, which will make it easier to follow.

Response:

1. It is really true as Reviewer suggestion. Detailed figures of corpus cavernosum catheterization have been added to figure file.

Special thanks to you for your good comments.

Reviewer #2:

Response to comment:

1. Whether unilateral cavernous nerve injury or bilateral cavernous nerve injury was carried out in this study needs to be clearly clarified.

2. The article mentioned in line 57 that Inhaled isoflurane anesthesia was used in all surgical procedures, while in line 71 it shown that Anesthetize rats by sodium pentobarbital (60 mg/kg) intraperitoneal injection, please be more clear.

Response:

1. Considering the Reviewer's suggestion, we have made it clear that bilateral cavernous nerve injury was carried out in this study. We have re-written this part to explain more details.

2. We are very sorry for our incorrect writing. Inhaled isoflurane anesthesia has been corrected to Sodium pentobarbital intraperitoneal injection anesthesia.

Special thanks to you for your good comments.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes but marked in red in revised paper.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.

Sincerely yours,

Zitaiyu Li