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Cover Letter

Dear Editor of the *Journal of Visualized Experiments (JoVE)*,

We take the liberty of submitting the manuscript of a paper entitled *"Functional and physiological methods of evaluating median nerve regeneration in the rat"* for publication in the ***Journal of Visualized Experiments (JoVE)***.

Arguably, using the median nerve (**MN**) of the rat, it is possible to adequately replicate peripheral nerve lesions in humans.[1-3] In this paper **we present a model of peripheral nerve lesion and repair in the rat**. In particular, we demonstrate in a detailed fashion the steps required to produce and repair different types of MN lesions in the rat. Moreover, we illustrate the use of different settings adequate to simulate postoperative physiotherapy. Finally, we show how to evaluate functional recovery of this nerve using several tests.

We strongly believe that **this model is particularly indicated to replicate the clinical scenario, facilitating extrapolation of results to the human species.**

We base this description in our groups' 15-year experience with the use of this model for both teaching and research purposes.[1,4] To make this subject more perceptible to a wider audience, we have included an original professional-drawn diagram illustrating the relevant anatomy of the MN in the rat.

We believe this paper will undoubtedly be of great interest to the readers of ***JoVE***, as the MN model has multiple advantages relatively to the most commonly used sciatic nerve model.

We very much hope you decide to publish it!

If you have any doubts or queries, please do not hesitate to contact us.

Thank you so much for your kind time and attention.

Yours sincerely.

The authors

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1 **TITLE:**
2 **Functional and Physiological Methods of Evaluating Median Nerve Regeneration in the Rat**
3
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38 **KEYWORDS:**
39 median nerve, nerve regeneration, peripheral nervous system, repair, rat, experimental model,
40 surgery, functional tests
41
42 **SUMMARY:**

43   Presented is a protocol to produce different types of median nerve (MN) lesions and repair in the
44   rat. Additionally, the protocol shows how to evaluate the functional recovery of the nerve using
45   several noninvasive behavioral tests and physiological measurements.
46
47   **ABSTRACT:**
48   The main goal of this investigation is to show how to create and repair different types of median
49   nerve (MN) lesions in the rat. Moreover, different methods of simulating postoperative
50   physiotherapy are presented. Multiple standardized strategies are used to assess motor and
51   sensory recovery using an MN model of peripheral nerve lesion and repair, thus permitting easy
52   comparison of the results. Several options are included for providing a postoperative
53   physiotherapy-like environment to rats that have undergone MN injuries. Finally, the paper
54   provides a method to evaluate the recovery of the MN using several noninvasive tests (i.e.,
55   grasping test, pin prick test, ladder rung walking test, rope climbing test, and walking track
56   analysis), and physiological measurements (infrared thermography, electroneuromyography,
57   flexion strength evaluation, and flexor carpi radialis muscle weight determination). Hence, this
58   model seems particularly appropriate to replicate a clinical scenario, facilitating extrapolation of
59   results to the human species.
60
61   Although the sciatic nerve is the most studied nerve in peripheral nerve research, analysis of the
62   rat MN presents various advantages. For example, there is a reduced incidence of joint
63   contractures and automutilation of the affected limb in MN lesion studies. Furthermore, the MN
64   is not covered by muscle masses, making its dissection easier than that of the sciatic nerve. In
65   addition, MN recovery is observed sooner, because the MN is shorter than the sciatic nerve. Also,
66   the MN has a parallel path to the ulnar nerve in the arm. Hence, the ulnar nerve can be easily
67   used as the nerve graft for repairing MN injuries. Finally, the MN in rats is located in the forelimb,
68   akin to the human upper limb; in humans, the upper limb is the site of most peripheral nerve
69   lesions.
70
71   **INTRODUCTION:**
72   Peripheral nerve lesions occur regularly as a result of trauma, infection, vasculitis, autoimmunity,
73   malignancy, and/or radiotherapy[1,2]. Unfortunately, peripheral nerve repair continues to present
74   clinically unpredictable and frequently disappointing results[3,4]. There is widespread consensus
75   that considerable basic and translational research is still needed to improve the prospect of those
76   affected[4-7].
77
78   The rat MN shows great similarities to that of humans[8,9] (**Figure 1**). Originating from the brachial
79   plexus in the axillary region, this nerve descends into the medial aspect of the arm, reaching the
80   elbow, and branching off to the majority of the muscles in the ventral compartment of the
81   forearm. The MN reaches the hand, where it innervates the thenar muscles and the first two
82   lumbrical muscles as well as to part of the rat's hand skin[9] (**Figure 1**).
83
84   Using the rat MN, it is possible to adequately replicate peripheral nerve lesions in humans[10-12].
85   This nerve has several potential research advantages relative to the customarily used sciatic
86   nerve. Because the MN is located in the forelimb of rats (akin to the human upper limbs), it can

87   be damaged experimentally with a much smaller impact on the rat's wellbeing, compared to the
88   sciatic nerve, which innervates a substantial portion of the pelvic limb[13]. Additionally, in humans
89   most clinical lesions occur in the upper limb, which corresponds to the rat's forelimb[10-12,14-16].
90
91   This paper shows how to produce different types of MN lesions in the rat. Moreover, different
92   ways to simulate postoperative physiotherapy are presented. Finally, tests to evaluate functional
93   recovery of the MN are described. There are multiple standardized strategies available to assess
94   motor and sensory recovery using an MN model of peripheral nerve lesion and repair, thus
95   permitting an easy comparison of results. The MN model is particularly suitable to replicate the
96   clinical scenario, facilitating extrapolation of results to the human species.
97
98   **PROTOCOL:**
99
100 All procedures involving animal subjects were approved by the Institutional Animal Care and Use
101 Committee and Ethical Committee at Nova University Medical School, Lisbon, Portugal
102 (08/2012/CEFCM).
103
104 **1. Median nerve surgery**
105
106 NOTE: Follow aseptic technique during the surgery. Use personal protective instruments (PPE)
107 and wear a sterile surgical gown[17]. Autoclave all the required surgical instruments prior to the
108 surgery (see the **Table of Materials**).
109
110 1.1. Use 12-week-old Wistar rats. Provide them with ad libitum food and water with 12 h light-
111 dark cycles 7 days prior to surgery. Prior to anesthesia, weigh the rat to determine the amount
112 of anesthetic required.
113
114 1.2. Anesthetize the rat with an intraperitoneal injection of a mixture of ketamine (40–80 mg/kg
115 body weight) and xylazine (5–10 mg/kg of body weight). Check for the depth of anesthesia by the
116 lack of response to a toe pinch and by observing of respiration rate throughout the entire
117 procedure[18,19]. Provide additional analgesia if a respiratory rate over 110 cycles/min or motor
118 response to toe pinching is observed[18,20].
119
120 1.3. Inject 1 mg/kg of meloxicam subcutaneously to provide preemptive analgesia[20,21].
121
122 1.4. To avoid corneal abrasion during the surgery, apply ophthalmic gel to both the eyes.
123
124 1.5. Use a depilatory cream to remove the hair over the medial aspect on the right. Once done,
125 wash with warm saline to remove the cream[17].
126
127 1.6. Lay the rat in supine position on a heating pad. Apply an iodine or chlorhexidine-based
128 surgical scrub over the operative site. Leave it for at least 15 s and then wipe with ethanol. Repeat
129 the application 3x. Ensure that the scrub is in contact with the skin for at least 2 min prior to
130 proceeding with the surgery.

131

132   NOTE: Contact the infection control authority at your research unit for alternative protocols to
133   prevent surgical site infection[19].

134

135   1.7. Drape the surgical area[17].

136

137   NOTE: Perform all procedures under strict aseptic conditions[19].

138

139   1.8. Incise the skin in the medial aspect of the right arm and pectoral region to the deep fascial
140   plane, using a number 15 scalpel blade. Carefully cauterize any bleeding vessels using an electric
141   cautery.

142

143   1.9. Carefully divide the brachial fascia, which presents as a whitish sheath covering the muscles,
144   using a thermocautery or a pair of blunt scissors, taking care not to damage the vascular and
145   nerve structures in the medial aspect of the arm.

146

147   1.10. Open a pair of scissors bluntly beneath the terminal insertion of the pectoralis major
148   muscle, in order to tease this muscle away from the underlying axillary artery and vein, as well
149   as from the terminal branches of the brachial plexus.

150

151   1.11. Divide the insertion of the pectoralis major muscle with an electric cautery. Expose and
152   section the pectoralis minor muscle.

153

154   1.12. Bluntly dissect the MN from the brachial vessels and from the ulnar nerve starting from the
155   axillary region up to the elbow. This allows exposure of the different terminal branches of the
156   brachial plexus, namely the median, the ulnar, the radial, the axillary and the musculocutaneous
157   nerves (**Figure 2**).

158

159   1.13. Separate different experimental groups as described below.

160

161   1.13.1. Create the *Sham* group by only dissecting the MN.

162

163   1.13.2. Create the *Crush* group by compressing the MN in the middle portion of the arm for 15 s
164   using a number 5 microsurgery forceps, or a similar instrument[22,23].

165

166   1.13.3. Create the *Excision* group using a pair of microsurgery scissors to excise a 10 mm-long
167   segment from the central part of the MN in the arm. Ligate the proximal stump of the nerve with
168   an 8/0 Nylon suture to prevent axonal growth.

169

170   1.13.4. Create the *Graft* group using the 10 mm-long-segment of the MN described in the latter
171   step and rotating it 180°. Suture the proximal and distal stumps of the sectioned MN to the nerve
172   graft using interrupted 10/0 Nylon stitches.

173

174   1.14. Close the skin wound using interrupted 5/0 Nylon stitches[10,24].

175

176   1.15. Provide postoperative analgesia with 7 mL of cherry-flavored acetaminophen mixed with

177   43 mL of tap water[25] to obtain a concentration of 4.48 mg/mL in 50 mL plastic water bottles made

178   available to the rats ad libitum for 3 days[25].

179

180   **2. Housing and physiotherapy**

181

182   2.1. Allow rats to have contact with the physiotherapy apparatuses 2–4 weeks before surgery, in

183   order to ensure an easier and faster adaptation to the exercise settings. Perform the exercises by

184   following the procedures detailed below.

185

186   2.2. Once a day, place each rat inside an individual physiotherapy sphere, and subsequently place

187   the sphere in a room with few obstacles. Let the rat ambulate and explore the room freely for

188   half an hour.

189

190   2.3. House the rats individually in solitary cages with incorporated running wheels to help them

191   exercise.

192

193   2.4. Form groups of 4–5 animals and house these groups in personalized cages. Personalize the

194   cages by including ladders, ropes, running wheels, and other environmental enriching elements.

195

196   2.5. Return individual rats to personalized cages the day after surgery.

197

198   2.6. Resume physiotherapy exercises 3 days after surgery.

199

200   **3. Functional tests**

201

202   3.1. One week before starting to perform the functional tests, familiarize the rats with the food

203   treats to be used as positive reinforcement. Provide this reinforcement after the successful

204   completion of each test, before and after surgery. After the initial training period of 3 weeks,

205   resume all tests 1 week after surgery.

206

207   3.2. Conduct the tests in the evening, when the rats are naturally more active. Resume tests 1

208   week after surgery.

209

210   3.3. Perform the Grasping Test by placing the rat on a grid and lift it by its tail, letting it grab the

211   grid with its forepaws[11,26]. Assign a "Positive" score if the rat can grab the grid with both

212   forepaws. Assign a "Negative" score if the rat cannot grab the grid with the injured paw.

213

214   NOTE: A positive Grasping Test indicates that the motor component of the MN is functioning[16,27].

215

216   3.4. Perform a Pin Prick Test[28,29].

217

218   3.4.1. Make a plastic platform with a 4 mm x 4 mm square grid pattern. Provide support to this

219   grid with a 21 cm long metallic frame.

221   3.4.2. Place the rat on the platform and cover the grid with a 15.5 cm x 15.5 cm x 11 cm
222   transparent plastic box. Wait for few minutes until normal activities (e.g., exploratory and major
223   grooming) subside.

225   3.4.3. Start the test when the rat is stationary and standing on its four paws.

227   3.4.4. With the help of a mirror, insert an aesthesiometer (e.g., number 4 Von Frey hair with a
228   bending force of 25 g) through the mesh and poke the palmar aspect of the forepaw in the skin
229   territory of the MN (**Figure 1**). Repeat the assessment 5x on each forepaw alternatively, waiting
230   a few seconds after each evaluation.

232   3.4.5. Check for the bending of the Von Frey filament[30] for the correct evaluation. Score the
233   withdrawal responses as follows: "0" for no withdrawal response, "1" if the rat slowly removes
234   the paw from the filament, "2" if the rat quickly responds to the stimulus and removes the paw
235   or licks the paw.

237   NOTE: If ambulation and biting of the filament are observed repeat the stimulus, because these
238   are considered ambiguous responses.

240   3.5. Training sessions

242   NOTE: Train the rats daily for 3 weeks before performing the surgery in the evening in a low-light
243   environment. The training sessions are recommended specially for the rope climbing, ladder
244   rung, and walking tracks tests. These can be done in the order presented before, starting with
245   the rope climbing test, ladder rung, and finally walking tracks test. Allow the same animal a few
246   minutes of rest before a new test.

248   3.5.1. During the first week, place the rat on the last third of the ladder/rope/corridor, close to
249   the box's entrance. Condition the animal to move towards the box opening by gently touching
250   and/or pulling the tip of its tail. Provide the rat a food treat once it enters the box, allowing it
251   some seconds of rest before repeating the test. Repeat it 5x each day for 5 days.

253   3.5.2. During the second week, place the animal on the second third of the ladder/rope/corridor
254   Repeat the steps in 3.5.1.

256   3.5.2. During the third week, place the rat on the bottom of the ladder/rope/corridor, on the
257   opposite side of box's entrance. Repeat the steps in 3.5.1, but only reward the animal when it
258   completes the test correctly.

260   3.6. Perform the Ladder Rung Test.

262   NOTE: This test is used for assessing forelimb strength, stepping, placing, and coordination[31].

263

264   3.6.1. Place the rat at the bottom of the ladder (120 cm x 9 cm x 2 cm with 18 steps 1.5 cm-thick,
265   spaced 4 cm apart) and gently touch the rat's tail. Ensure that the ladder is placed at an inclination
266   of 10˚ and leads to a 13.20 cm x 11 cm opening on a dark wooden 31.5 cm x 35 cm x 35 cm box.

267

268   3.6.2. Start the timer once the rat starts climbing the ladder and stop the timer once the rat's
269   snout crosses the box's entrance.

270

271   3.6.3. Record the time and repeat the test 3x, each separated at least by a 1 min interval.

272

273   3.7. Rope Climbing

274

275   NOTE: This test is used for assessing grasping strength, which is dependent on MN recovery[32].

276

277   3.7.1. Put the rat at the bottom of the rope and persuade it to climb by gently touching its tail.
278   Start the timer once the animal starts climbing and stop it the moment the rats' snout crosses
279   the platform's entrance.

280

281   3.7.2. For each test, record the time taken to climb to the platform and the number of slips of
282   the injured paw while the rat climbs up the rope. Consider the test valid if the animal does not
283   hesitate during the task or does not stop climbing. Provide the rat with a snack after performing
284   the task correctly.

285

286   3.7.3. Record the time and repeat the test 3x, each separated at least by a 1 minute interval.

287

288   3.8. Walking Tracks

289

290   NOTE: This test is used for the evaluation of forelimb motor recovery[33,34].

291

292   3.8.1. Set up an apparatus consisting of a confined walkway 16.5 cm in height x 8.7 cm in width x
293   43 cm in length. Ensure that this leads to a rectangular 8.8 cm x 8.2 cm opening in one of the
294   walls of a black wooden 23 cm x 36 cm x 28 cm box. Include a vertical sliding door to close the
295   box's entrance rapidly. Include a removable top to be used for retrieving the rat[33,34].

296

297   3.8.2. Place a piece of graph paper on the corridor's floor. Grab the rat by its tail and let it hold a
298   painting brush soaked in methylene blue. Position the rat at the entrance of the corridor to make
299   it walk inside the box. Remove the graph paper from the corridor's floor and repeat the test until
300   a good representative impression of both forepaws is obtained.

301

302   3.8.3. From the obtained prints, select one with clear successive forepaw prints, photograph
303   them in a tiff or jpeg format, and measure the following parameters using the open access
304   software FIJI[35].

305

306   NOTE: First, calibrate each image using the markings in the graph paper (**Analyze | Set scale**)

307 Second, convert each image to an 8-bit format (**Image | Type | 8-bit**). Subsequently, use the
308 **Rectangular Selection Tool** to select the paw print. Crop this portion of the image (**Image | Crop**).
309 In each image, highlight the pawprints and remove the background by thresholding the image
310 (**Image | Adjust | Threshold**).
311
312 3.8.3.1. Measure the stance factor by measuring the paw impression area. Use the **Rectangular**
313 **Selection Tool** to select the paw print and press **Control + M**.
314
315 3.8.3.2. Measure the print length factor by measuring the longest length of the paw impression
316 (for steps 3.8.3.2–3.8.3.6, use the **Straight-Line Selection Tool** for selecting the two most distant
317 points and press **Control + M**).
318
319 3.8.3.3 Measure the finger spread factor by measuring the widest width of the paw impression.
320
321 3.8.3.4. Measure the intermediate finger spread factor by measuring the widest width between
322 the second and third fingers.
323
324 3.8.3.5. Measure the stride length by measuring the distance between homologous points of
325 successive paw impressions on a given side.
326
327 3.8.3.6. Measure the base of the support by measuring the perpendicular distance between the
328 central portion of the paw impression and the direction of movement[29,33,36].
329
330 NOTE: Perform the last two measurements in two pairs of representative consecutive bilateral
331 paw impressions[33].
332
333 **4. Physiological measurements**
334
335 4.1 Infrared Thermography (IRT)[37-39].
336
337 4.1.1. Ensure that the temperature of the room where the measurements will be performed is
338 between 18 °C–25 °C using a normal digital hydrothermometer with a thermal resolution of 0.1
339 °C. Ensure that no significant heat sources (e.g., computers or refrigerators) are present.
340
341 4.1.2. Acclimate the rats by bringing them to the evaluation room 2 h prior to the evaluation.
342 Before starting the experiment, anesthetize the rat as described above (steps 1.3–1.6) or
343 following the institution's protocol. Check for the lack of the response to a toe pinch before
344 starting the experiment.
345
346 4.1.3. Switch on the infrared thermographic camera 15 min prior to the acquisition and do not
347 turn it off during the evaluations. Set the emissivity parameter of the camera to match that of
348 the rat's skin ($\varepsilon = 0.98$)[37,40,41].
349
350 4.1.4. Place the rat on its dorsum on a clean and stable surface with a polyethylene sponge.

351   Ensure that there are no reflective materials and other possible sources of artefacts. Fix its
352   forepaws carefully in supination with double face glue tape. Insert a digital thermometer 2 cm
353   inside the rectum to monitor the rat's central temperature during all evaluations.
354
355   4.1.5. Hold the thermographic camera at a 90° angle and 30 cm distance from the rat. Focus the
356   camera on the whole animal's body. Obtain three infrared thermography images spaced 30 s
357   apart.
358
359   4.1.6. Transfer the acquired thermograms to a computer and analyze them using analysis
360   software. Define the temperature of the plantar surface of both forepaws using a fixed
361   rectangular region of interest (e.g., 9 x 11 pixels) in the plantar territory of the MN, for example
362   in the center of the first metacarpal foot pad (**Figure 1**). Using the free FLIR Tools software, select
363   the thermography by double clicking on it. On the left tools bar select the button "**Add box**
364   **measurement tool**" and draw a rectangle of 9*11 pixels over the plantar area of both forepaws.
365   While adjusting the rectangle its dimension in pixels can be confirmed. Perform it on both
366   forepaws. On the right side of the image find the maximum, minimum, and average
367   temperatures.
368
369   4.1.7. Over the previously drawn ROI, right click and select export. The mean, maximum, and
370   minimal temperatures, as well as a matrix of temperatures of the ROI are then exported to a .csv
371   document. These data can later be explored using data analysis software.
372
373   4.2. Electroneuromyographic (ENMG) Evaluation
374
375   4.2.1. Set up an electrical stimulator. Tape a pair of disposable acupuncture needles (0.25 mm x
376   25 mm) with a negligible impedance [<1 Ω]) and 25 mm between them to create electrodes for
377   stimulation. Now connect the stimulator and the electrode to a data acquisition unit to take the
378   incoming signals and convert them into digital signals that can be processed with the computer
379   software.
380
381   4.2.1. Perform the evaluations in the same room, and always under the same controlled
382   environmental conditions[42-44]. Pinch the forepaw to ensure that the rats are deeply anesthetized
383   before starting data acquisition.
384
385   NOTE: Deep anesthesia is of paramount importance to minimize variability associated with
386   spontaneous voluntary and/or involuntary movements by the rats[43].
387
388   4.2.2. Expose the MN on both sides under the surgical microscope as described in steps 1.8–1.13.
389   Use a number 15 scalpel blade to prolong the brachial incisions into the forearms with a ventral
390   midline incision.
391
392   4.2.3. Expose the superficial aspect of the flexor digitorum sublimis muscles by bluntly separating
393   the overlying antebrachial fascia using iris scissors. Insert the ground needle in the quadriceps
394   femoris muscle of the left hindlimb to connect the signal ground plug.

395

396 4.2.4. Start with the right forepaw and place the recording electrodes into the flexor digitorum
397 sublimis muscle belly of the forepaw and the stimulation electrode proximal to the lesion site in
398 the MN. Moisten these electrodes with saline.

399

400 4.2.5. Ensure that the software is set as follows: channel input port 1 (CH1) – stimulator to 0–10
401 V; and channel input port 2 (CH2) - EMG to 30–1,000 Hz. Start by choosing a stimulation
402 amplitude of 10 mV and record the compound muscle action potentials CMAPs sample rate of
403 50 kHz for a duration of 40,000 ms. Gradually increase the stimulation amplitude in 10 mV steps
404 until reaching 2,000 mV. Repeat the same for the left paw[42-44].

405

406 NOTE: The signal is amplified to 1,000x and filtered using a 30–1,000 Hz band. The stimulation
407 output is set for a single pulse with a duration of 1 ms[42-44].

408

409 4.2.6. Open the recorded file in the recording apparatus software.

410

411 NOTE: By default, the screen will show tow windows on top in red, the stimulator pulses, and
412 below in blue the recorder ENMGs. Sliding the horizontal scroll bar underneath the time scale
413 allows visualization of the full record. The two main tools, **Zoom tool** and **I-Beam tool**, are on the
414 bottom right side of the panel. Using the **Zoom tool**, it is possible to optimize the visualization of
415 the CMAPs and explore the graphics. To guarantee a good fitting on the visualization screen, it
416 may be necessary to adjust the display after zooming. To do so, select **Display|Autoscale**
417 **Waveforms**. The **I-beam tool** allows the selection of specific areas of the graphs and performance
418 of the desired measurements. On top of the graphs, there are three small windows where the
419 measurements are displayed. **P-P** shows the average amplitude value of the selected area in volts
420 (both in the stimulator record and in ENMGs), whereas **Delta-T** shows the time interval of that
421 selection.

422

423 4.2.7. Measure the parameters from the compound muscle action potential (CMPAs, described
424 in **Table 1**) using the homonymous measurement tools from the software plug in "*Toolbox for*
425 *unsupervised classification of MUAPs and action potentials in EMG*"[45].

426

427 4.2.8. For each rat, determine a minimal value of stimulation voltage after which the CMAPs
428 amplitude does not increase further. Start from a 0.05 mV stimulus, and give successive
429 increasing stimuli in incremental voltages of 0.05 mV.

430

431 4.2.9. Apply a stimulus 20% above this voltage in order to obtain a supramaximal stimulation
432 value.

433

434 4.2.10. After the latter value is determined and the corresponding stimulus applied, record the
435 next CMAPs parameters.

436

437 4.3. Flexion strength evaluation

438

439   4.3.1. Use the same stimulator and stimulation electrodes to electrically stimulate the MN as in
440   step 4.2. Set the input channel CH1 as the stimulator (0–10 V) and the output settings for stimuli
441   duration of 30 s with pulses of 1 ms duration and 1 Hz frequency. Link a dynamometer, with a
442   resolution of d = 0.001 N to a computer.
443
444   NOTE: Real time visualization of data can be obtained by building a plot of force per time (N/s)
445   using the software previously installed on the computer and linked to a dynamometer[46].
446
447   4.3.2. Place the rat as described in step 4.1.4. Place a 5/0 silk suture loop through the second
448   interosseous space of both forepaws. Attach the suture loop to the dynamometer's hook and the
449   forepaw aligned with the dynamometer without putting excessive strain on the suture line.
450
451   4.3.3. Fix the contralateral paw with tape to avoid spurious movement interferences in the
452   dynamometer readings.
453
454   4.3.4. Set the dynamometer to zero by clicking on the **ZERO** button.
455
456   4.3.5 Adjust the stimulator to a supramaximal amplitude stimulation of 1.5 V by adjusting the
457   voltage knot.
458
459   4.3.5. On the PC, open the software AFH-01. Open the separator "**Device**" and select the device
460   **FH5**. Create a new file ("*Measurements1*" is the name given by default) and rename the file.
461
462   4.3.6 Place the electrode on the proximal part of the MN, click on **play** at the bottom of the
463   program, record the pull on the dynamometer for 30 s.
464
465   4.3.7. Import the values obtained to a data analysis software. Calculate the maximum and
466   average force values and the area under the curve (AUC) for the strength x time graph for each
467   evaluation.
468
469   4.3.8. Repeat for the left forepaw.
470
471   4.4. Muscle weight
472
473   4.4.1. Euthanize the rats under general anesthesia by exsanguination[47,48].
474
475   4.4.2. Harvest the flexor carpi radialis muscle from both forearms, dissecting the muscle from its
476   origin until its distal tendon insertion, using a number 15 scalpel blade.
477
478   4.4.3. Weigh the muscles with a precision scale[9,49].
479
480   **REPRESENTATIVE RESULTS:**
481
482   A total of 34 rats were randomly divided into the following groups: *Sham* (n = 17), *Excision* (n =

483   17), and *Nerve Graft* (n = 10) for the operation. All rats survived surgery and the postoperative
484   period uneventfully. One week after surgery and for the subsequent 100 days, all animals
485   underwent the functional tests described above once a week. The representative results of each
486   of these tests are described below.

487

488   **Grasping Test**
489   The percentage of rats with a positive response in the grasping test was highest for the *Sham*
490   group. This value gradually increased over time in rats from the *Crush* and *Nerve Graft* groups
491   (**Figure 3**).

492

493   **Pin Prick Test**
494   Rats from the *Sham* group had the best scores in the cumulative pin prick test relative to rats
495   from the *Nerve Graft* group. Both had better scores than the rats in the *Excision* group (**Figure 4**).

496

497   **Ladder Running Test**
498   The rats' velocity in the ladder running test was highest in the *Sham* group than in the rats
499   submitted to MN lesion. Among the latter, the time to run the ladder tended to decrease over
500   time, paralleling MN recovery (**Figure 5**)**.**

501

502   **Rope Test**
503   As in the ladder running test, the time the rats took to climb the rope was shorter in the *Sham*
504   group compared to the groups in which the MN was injured. The rats' speed in this test increased
505   when the MN was allowed to recover (**Figure 6**).

506

507   **Walking Track Analysis**
508   Analysis of walking tracks tended to show changes in the morphology of paw prints (**Figure 7**).
509   These changes were often more pronounced in crushing injuries than in segmental nerve
510   lesions[50].

511

512   **Infrared Thermography**
513   Thermography was useful when examining temperature differences between the forepaws in
514   the first 30 days after surgery. Temperature differences were more noticeable in rats with a more
515   severely injured MN, such as in those from the *Excision* group (**Figure 8** and **Figure 9**).

516

517   **Electroneuromyography**
518   **Table 1** summarizes the biological importance of the electroneuromyography measurements,
519   providing representative results for the different experimental groups. Various patterns were
520   observed with electroneuromyography. A normal CMAP was typical of a rat from the *Sham*
521   group, while a polyphasic CMAP was associated with a variable degree of lesion of the MN, as in
522   the *Crush* and in the *Nerve Graft* groups (**Figure 10**). In the *Excision* group, no CMAPs were
523   observed.

524

525   **Wrist Flexion Strength**
526   Given that wrist flexion is mainly dependent on the MN, this test was used to evaluate motor

527 recovery in this nerve's territory. Wrist flexion strength was closest to normal when recovery was
528 maximal (**Figure 11**)**.**

530 **Muscle Weight and Morphology**
531 The weight and morphology of the flexor carpis radialis muscle were dependent on MN recovery,
532 as this muscle is innervated exclusively by the MN[9,10]. Thus, normal weight and morphology were
533 observed in the *Sham* group. A loss of weight and muscle trophism was observed in the *Crush*,
534 *Nerve Graft*, and *Excision* groups (**Figure 12**).

536 **FIGURE AND TABLE LEGENDS:**
537 **Figure 1: Schematic representation of the anatomy of the median nerve of the rat.** (**1**) Origin
538 and termination of the median nerve in the rat brain (green area = primary motor area; blue area
539 = primary sensory area). (**2**) Transverse section of the spinal cord at C7 segment level; (**3**) Axillary
540 nerve; (**4**) Musculocutaneous nerve; (**5**) Radial nerve; (**6**) Median nerve; (**7**) Ulnar nerve; (**8**)
541 Medial cutaneous branch of the arm; (**9**) Medial cutaneous branch of the forearm; (**10**) Axillary
542 artery; (**11**) Brachial artery; (**12**) Median artery; (**13**) Superficial radial artery; (**14**) Ulnar artery;
543 (**15**) Motor branch of the median nerve to the pronator teres muscle; (**16**) Motor branch of the
544 median nerve to the flexor carpis radialis muscle; (**17**) Motor branch of the median nerve to the
545 flexor digitorum superficialis muscle; (**18**) Motor branch of the median nerve to the flexor
546 digitorum profundus muscle; (**19**) Sensory branch of the median nerve to the thenar region; (**20**)
547 Common palmar artery of the first interosseous space; (**21**) Radial palmar digital artery of the
548 first digit; (**22**) Motor branch of the median nerve to the thenar muscles; (**23**) Palmar arterial
549 arch; (**24**) Radial palmar digital nerve of the first digit; (**25**) Ulnar palmar digital nerve of the first
550 digit; (**26**) Common palmar artery of the third interosseous space; (**27**) Motor branches of the
551 terminal divisions of the median nerve to the first three lumbrical muscles; (**28**) Ulnar palmar
552 digital nerves of the second, third, and fourth digits; (**29**) Ulnar palmar digital arteries to the
553 fourth and fifth digits; (**30**) Radial palmar digital nerves of the second, third, and fourth digits;
554 (**31**) Radial palmar digital artery of the fifth digit; (**32**) Skin territory of the median nerve in the
555 forepaw (blue-shaded region).

557 **Figure 2: Photograph of the right forelimb of the rat showing the surgical anatomy of the**
558 **median nerve in the arm and axillary regions.** Cr, cranial; Me, medial

560 **Figure 3: Percentage of rats with a positive grasping test in the different experimental group**
561 **over a period of 100 days after surgery.**

563 **Figure 4: Nociception evaluation using cumulative pin prick test results in the operated forepaw**
564 **normalized to the contralateral paw in the different experimental groups.** Vertical bars
565 represent 95% confidence intervals. Horizontal lines in the upper part of the figure indicate
566 statistically significant differences between experimental groups, ***p<0.001.

568 **Figure 5: Average speed in the ladder running test in the different experimental groups.** Vertical
569 bars represent 95% confidence intervals. Asterisks in the upper portion of the figure indicate
570 statistically significant differences between groups, *p<0.001.

571

Figure 6: Average climbing velocity in the rope test in the *Sham* and *Excision* groups. Vertical bars represent 95% confidence intervals. Asterisks in the upper portion of the figure show statistically significant differences between groups, *p<0.05; **p<0.01.

Figure 7: Walking track parameters in the different experimental groups. Values on the operated limb are expressed as percentages of means normalized to the contralateral limb. (**A**) Stance factor; (**B**) Print length; (**C**) Finger spread factor; (**D**) Intermediate finger spread factor; (**E**) Stride length; (**F**) Base of support. Vertical bars represent 95% confidence intervals. Horizontal lines in the upper portion of the figure indicate statistically significant differences between experimental groups. D30, D60, D90 = 30, 60, and 90 days after surgery, *p<0.05; **p<0.01; ***p<0.001.

Figure 8: Mean temperature difference registered by infrared thermography. The box plots represent the temperature difference between the palmar region of the median nerve on the operated side (right side) and the contralateral side (left) in the *Sham* (n = 17) and *Excision* (n = 17) groups, *p<0.05; **p<0.01.

Figure 9: Typical infrared thermography pattern of an animal from the excision group during the first 45 days after surgery.

Figure 10: Typical patterns of Compound Muscle Action Potentials (CMAPs) from an animal from the *Sham* and *Nerve Graft* groups 90 days after surgery.

Figure 11: Evaluation of wrist flexion strength on both forepaws 90 days postoperatively in different experimental groups. Wrist flexion strength was assessed using the area under the curve (AUC) over a time period of 30 s and using supratetanic stimulation. Vertical lines denote 95% confidence intervals. Horizontal lines in the upper portion of the figure highlight statistically significant differences between groups, **p<0.01.

Figure 12: *Flexor carpi radialis* muscle weight and macroscopic appearance 100 days after surgery. (**A**) Box plots depicting the normalized *flexor carpi radialis* muscle weight in different experimental groups, **p<0.01; ***p<0.001. (**B**) Photographs of the muscles on the right and left sides in the *Sham* and *Excision* experimental groups.

DISCUSSION:

This paper presents a protocol to create different types of MN lesions and repair in the rat. Additionally, it illustrates how to evaluate the functional recovery of this nerve using several noninvasive behavioral tests and physiological measurements.

Notably, several of the functional tests described in this paper, namely the Ladder Running Test and the Rope Test, are significantly dependent on the rat's willingness to perform the task with the expectation of obtaining the food reward[51-53]. It should be noted that certain rat strains are more amenable to training and performing reproducibly in this type of tests[51-53]. For example, Lewis rats perform poorly in these tests both in the training phase and subsequently[51-53].

Rat housing should permit ample freedom of movement in agreement with their natural exploratory behavior, in addition to allowing experimental animals to get familiar with some of the elements present in the functional tests[19]. Therefore, different forms of housing allowing higher freedom of movement are shown. The big cages are personalized with enrichment elements that are later used in the functional tests (e.g., ropes and ladders).

Arguably, these enriching elements as well as the cages with incorporated running wheels and the individual training spheres provide a form of postoperative physiotherapy similar to that offered to human patients operated on the peripheral nervous system[10].

Significantly, although some authors advocate dissecting the subcutaneous tissues and muscle fasciae bluntly or by clean cutting with a number 15 scalpel, the use of thermocautery when dissecting these structures is recommended to minimize the risk of postoperative hematoma.

It should be noted that numerous tests have been devised to test different aspects of peripheral nerve repair in the rat, namely axonal regeneration, target reinnervation, and functional recovery, some of which are beyond the scope of this study[29,54-56]. For example, kinematic analysis[29,36,55] and histomorphometric assessment[29,36,57] are widely employed by multiple authors. Additionally, several of these tests involve variations to maximize efficiency and/or reproducibility[54]. For example, mechanical algisemetry (i.e., evaluation of responses to mechanical painful stimuli) can be assessed qualitatively using a given von Frey filament, as described in the present paper, or semiquantitatively using successively stronger von Frey filaments, or even quantitatively using electronic devices that apply increasing pressures until a withdrawal response is observed[30,54].

Similarly, although several authors use walking track analysis to evaluate forelimb nerve repair in the rat, other authors argue that single MN lesions frequently fail to produce reproducible changes in pawprints[10,58,59]. Furthermore, some have stated that these changes may not be proportional to muscle recovery[10,60]. Bearing this in mind, some researchers have advocated the use of walking track analysis in the forepaw mainly when assessing recovery after crushing neve lesions rather than after segmental nerve reconstruction[10,50,61].

The Grasping Test is widely used to evaluate motor recovery of the muscles controlled by the

651 MN[16,27]. To guarantee uniformity and reproducibility of the data obtained with this test, applying
652 the Grasping Test using the well-established methodology proposed by Bertelli et al.[16] is
653 recommended. However, the current protocol differs in that it does not routinely immobilize the
654 contralateral paw to prevent undue stress[11,27]. It should also be noted that other authors, after
655 immobilizing the uninjured paw, quantitatively assess the Grasping Test using a dynamometer or
656 a scale[27,56]. However, this quantitative evaluation may be affected by the strength the researcher
657 applies to the rat's tail[26]. Furthermore, it is difficult to distinguish between the strength
658 generated by the digital flexor muscles (solely innervated by the MN in the rat and the object of
659 the Grasping Test[9]) from the strength produced by the wrist flexors, which include the flexor
660 carpi ulnaris that receives its innervation from the ulnar nerve[9,10,27]. In order to try to circumvent
661 these potential biases, this protocol uses an ordinal scale similar to the Medical Research Council
662 Scale commonly used to grade muscle strength in humans[10,11,62]. Alternatively, other authors
663 have described detailed assessment of grasping using video analysis and a video-based scoring
664 system[11,63].
665
666 A potential disadvantage of using the MN compared to the sciatic nerve is that a greater amount
667 of information is available regarding the latter nerve. This, in turn, can make comparison of data
668 obtained with the MN with that of prior experimental works more difficult[46,48,64]. Additionally,
669 the smaller size of the MN compared to the sciatic nerve makes surgical manipulation more
670 challenging[8,12,27,56,65].
671
672 Contrary to the methodology described in this paper, the electroneuromyography evaluation can
673 be performed using transcutaneous monopolar electrodes placed in the arm and thenar
674 regions[51]. Despite being less invasive, this method carries the risk of potential confusion due to
675 the possibility of costimulation of the ulnar nerve in the arm region[9,51].
676
677 Most authors concur that not all tests used in the rat provide concordant results, as peripheral
678 nerve repair depends on a complex array of factors, comprising neuron survival, axonal
679 elongation and pruning, synaptogenesis, successful recapture of the denervated sensory organs
680 and motor units, and brain plasticity[7,10,50,66,67].
681
682 Finally, it should be noted that a significant caveat of rodent models is that rat peripheral nerves
683 are much closer to their end organs and have much smaller cross-sectional areas than the
684 homologous human structures. However, this size difference guarantees faster experimental
685 data in rodents, and better overall results in rats in comparison to humans are to be expected[68].
686 Indeed, several authors warn that care must be used when trying to extrapolate experimental
687 data obtained in peripheral nerve repair using rodents to humans[7,69]. Primate models are
688 considered more comparable[70]. Nevertheless, their use is associated with vexing ethical,
689 logistical, and budget constraints[71].
690
691 Even though the sciatic nerve is the most commonly used nerve in peripheral nerve research, the
692 rat MN presents multiple advantages. For example, MN lesions are associated with a smaller
693 incidence of joint contractures and automutilation of the affected paw[11,12,16,56]. Significantly,
694 autotomy subsequent to sciatic nerve transection afflicts 11–70% of rats. This may make current

695   evaluations like the sciatic index impossible[14]. This, in turn, makes the estimate of the number of
696   animals required to obtain a given statistical power cumbersome[15].
697
698   In addition, as the MN is shorter than the sciatic nerve, nerve recovery is observed sooner[58,72-76].
699   Furthermore, the MN is not covered by muscle masses, making its dissection technically easier
700   than that of the sciatic nerve[16]. Additionally, the MN has a parallel path to the ulnar nerve in the
701   arm. Hence, the ulnar nerve can easily be used as nerve graft for repairing MN injuries. Finally, in
702   humans, most peripheral nerve lesions occur in the upper limb, which further supports the use
703   of this nerve in the rat[77,78].
704
705   Arguably, rodents are the experimental animals most commonly used in the realm of peripheral
706   nerve repair[48,79]. As shown, the rat MN is a convenient model of peripheral nerve lesion and
707   repair. In fact, there are multiple standardized strategies available to assess motor and sensory
708   recovery, permitting an easier comparison of results[36,46,60,80-82]. Many of these methods are
709   noninvasive, allowing for daily assessment.
710
711   Moreover, physiotherapy is part of the standard of care of patients recovering from peripheral
712   nerve injuries. As demonstrated in this paper, there are multiple strategies to provide a
713   postoperative physiotherapy-like environment to rats submitted to MN injuries[4,5]. Hence, this
714   model is particularly suitable to replicate the clinical scenario, facilitating extrapolation of results
715   to the human species[12,27,48,56,58,83].
716
717   As shown in this paper, multiple standardized strategies are available to assess motor and
718   sensory recovery in the MN model of the rat. The majority of these are noninvasive procedures,
719   allowing frequent assessment. Moreover, as most peripheral nerve lesions in the human species
720   occur in the upper limb, the mentioned experimental physiotherapy settings can more aptly
721   mimic recovery in the clinical context. Arguably, this can facilitate extrapolation of results to the
722   human species, further validating the use of this nerve in the rat.
723

732

733   **DISCLOSURES:**
734   The authors have nothing to disclose.
735

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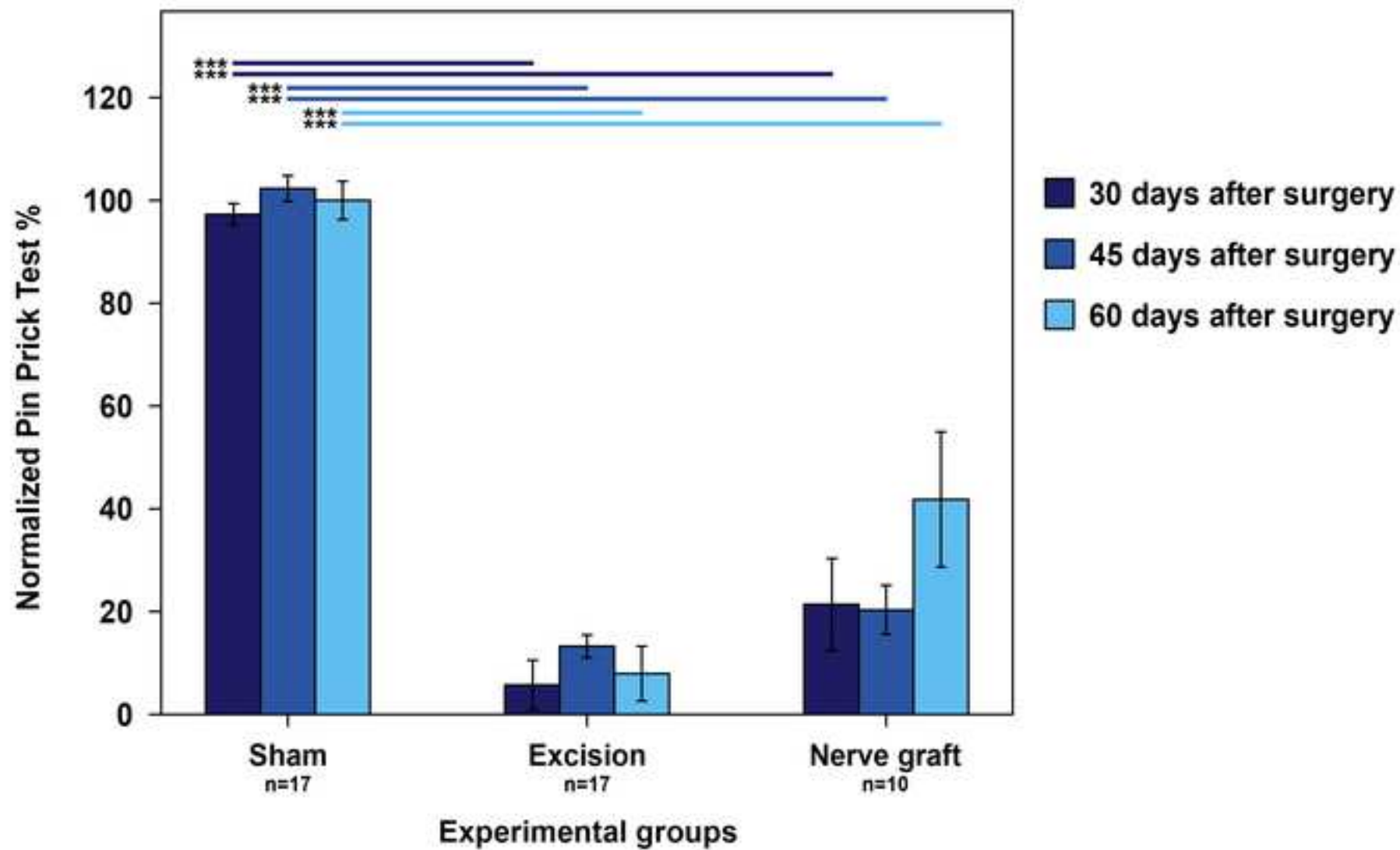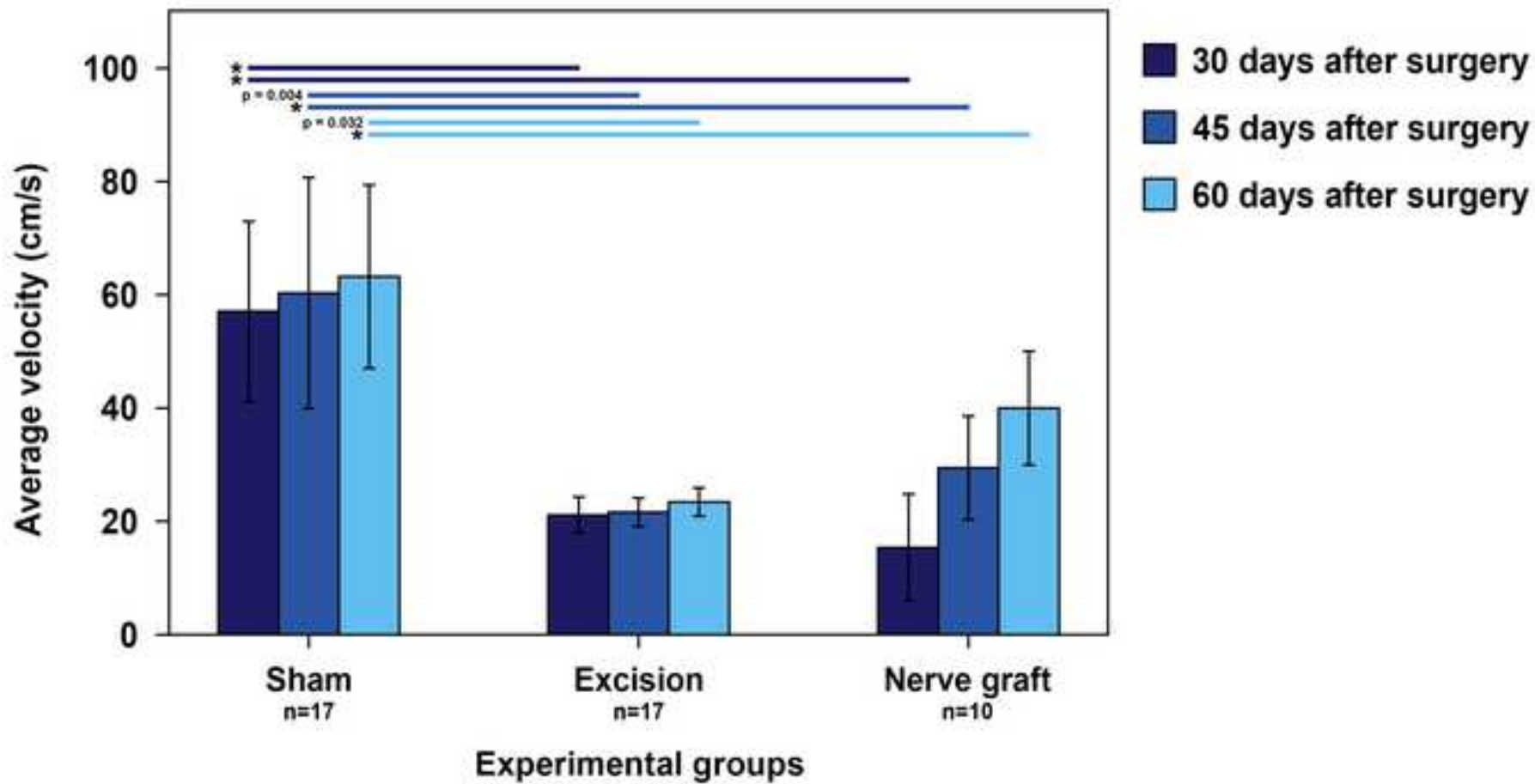943

Figure 1

Filipe Franco
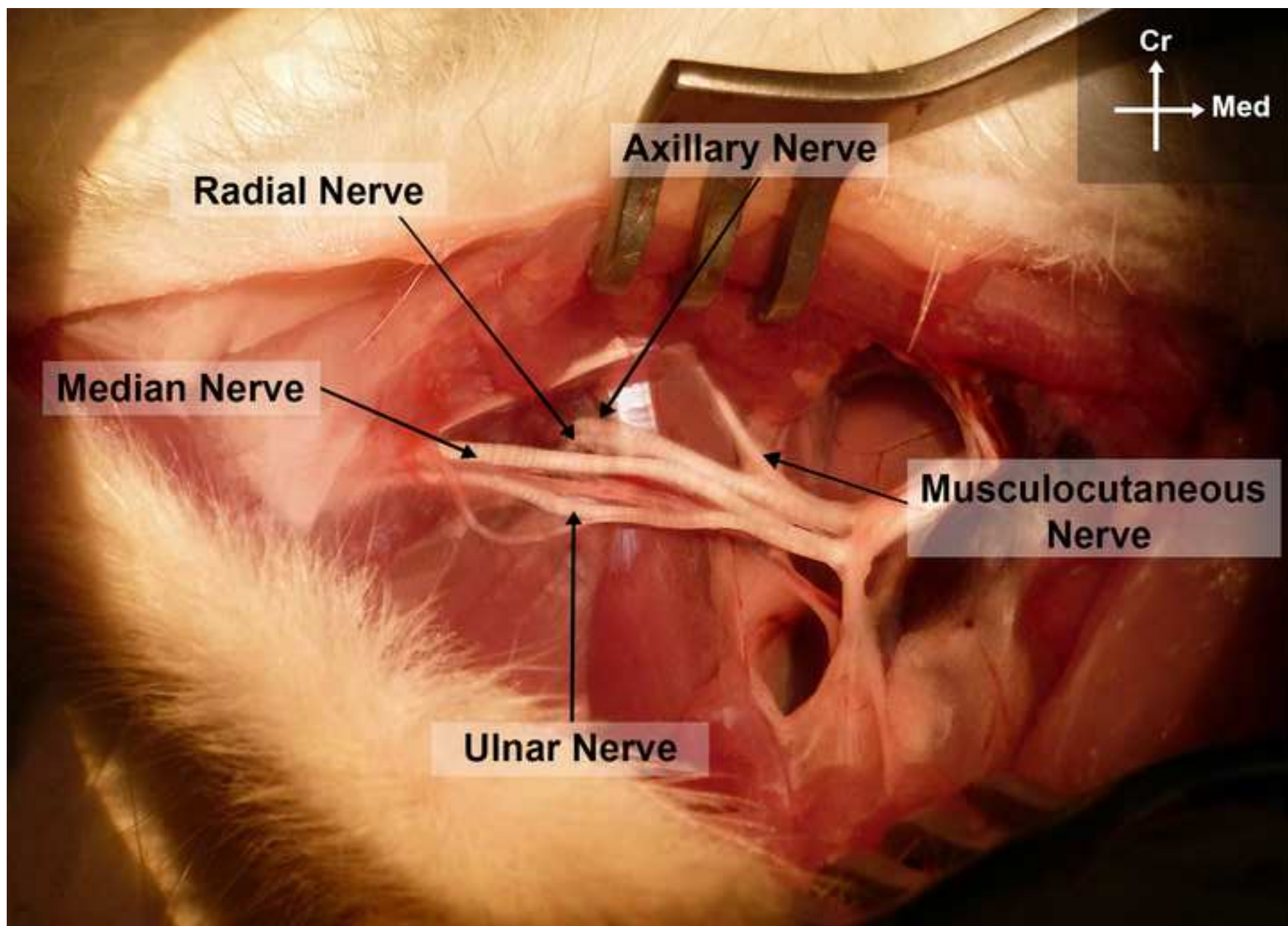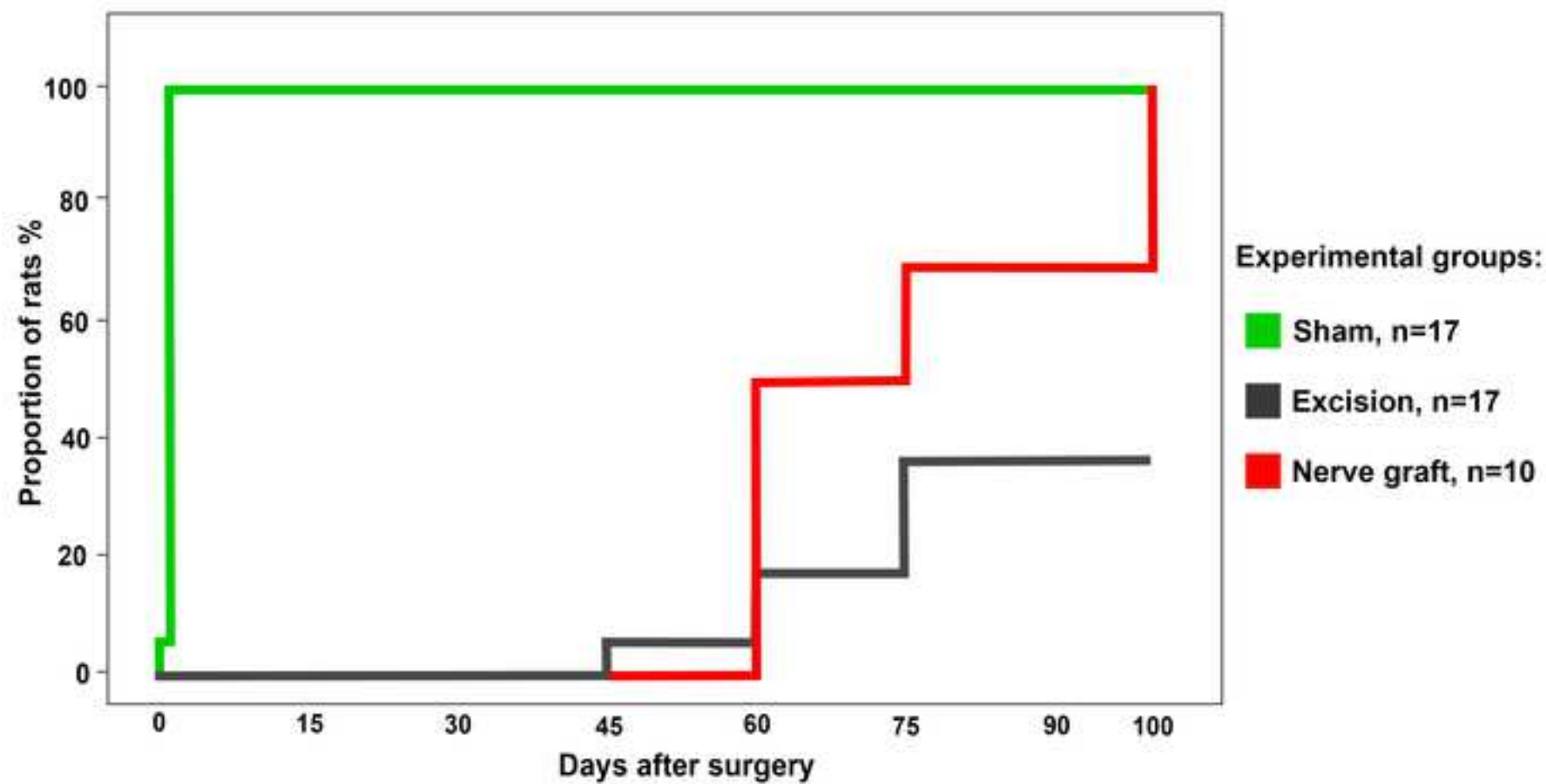
Figure 2

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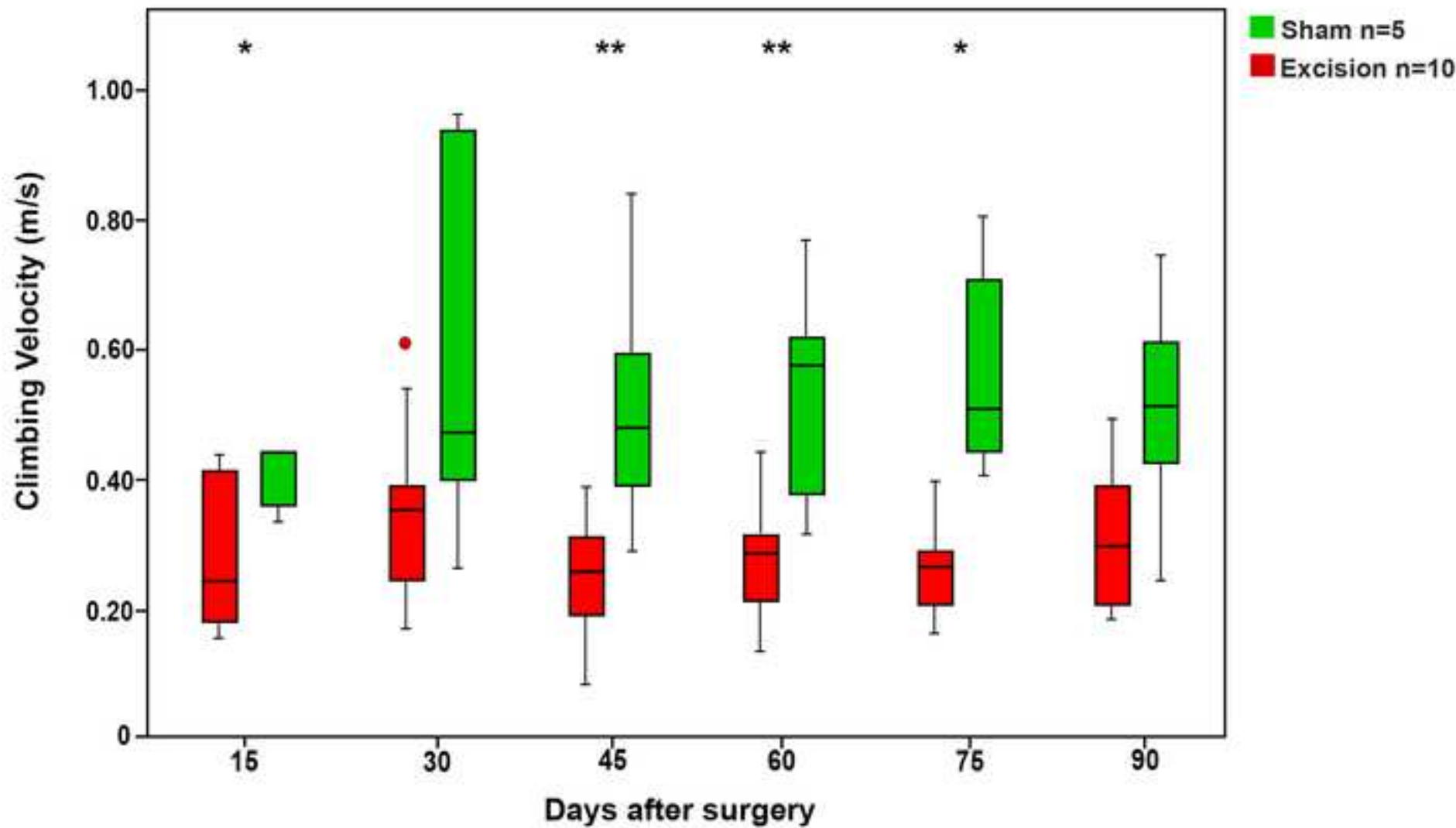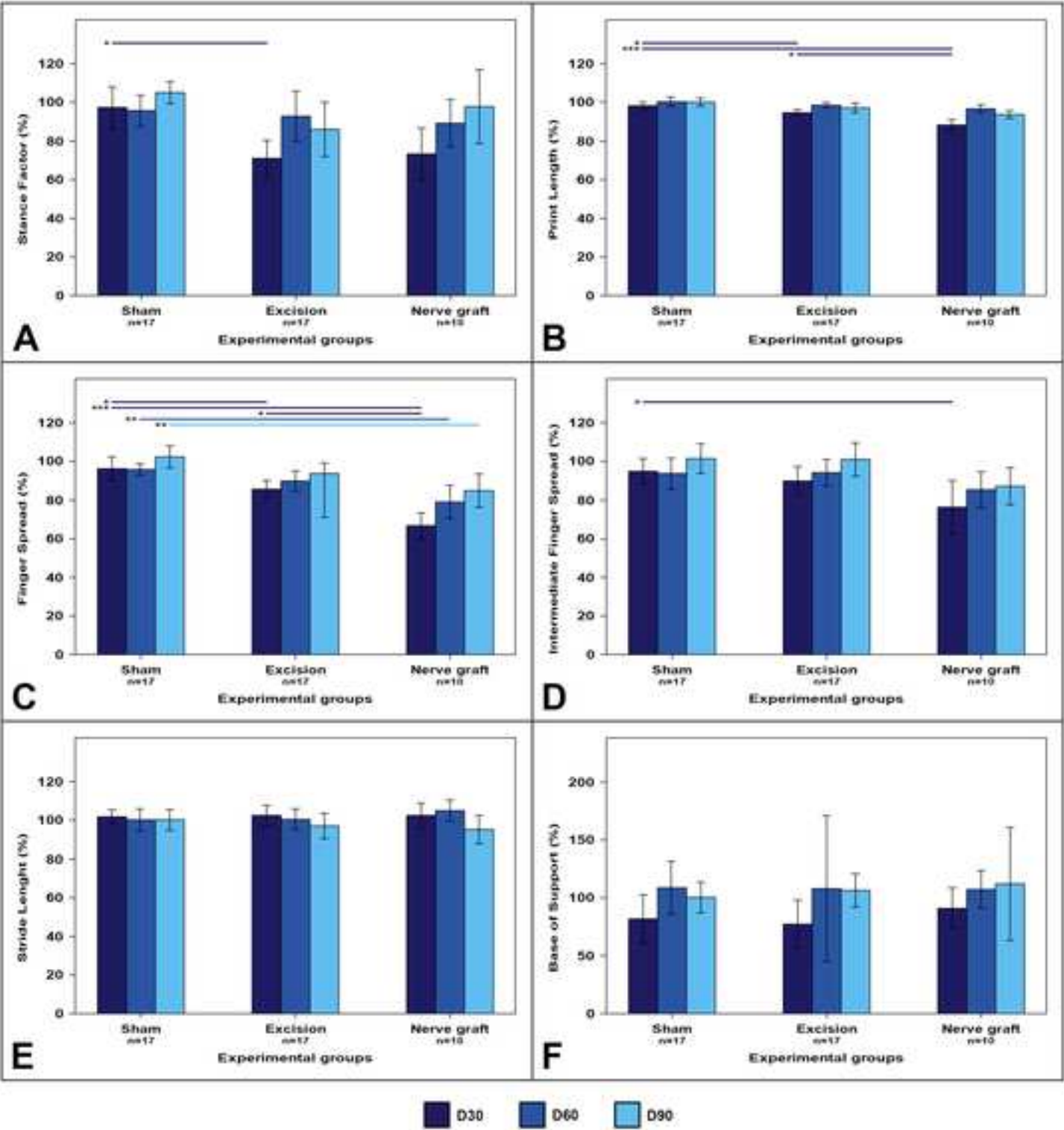
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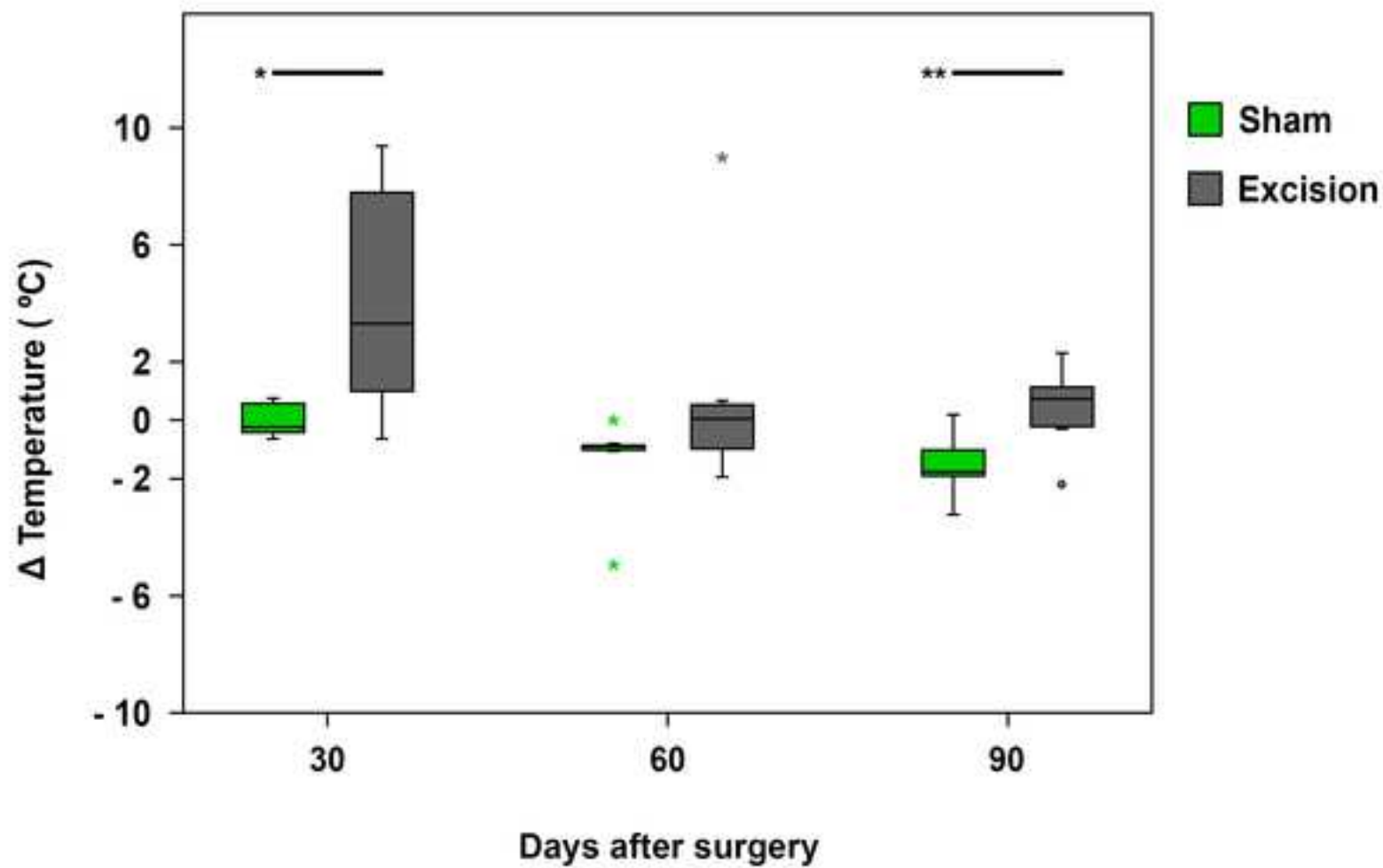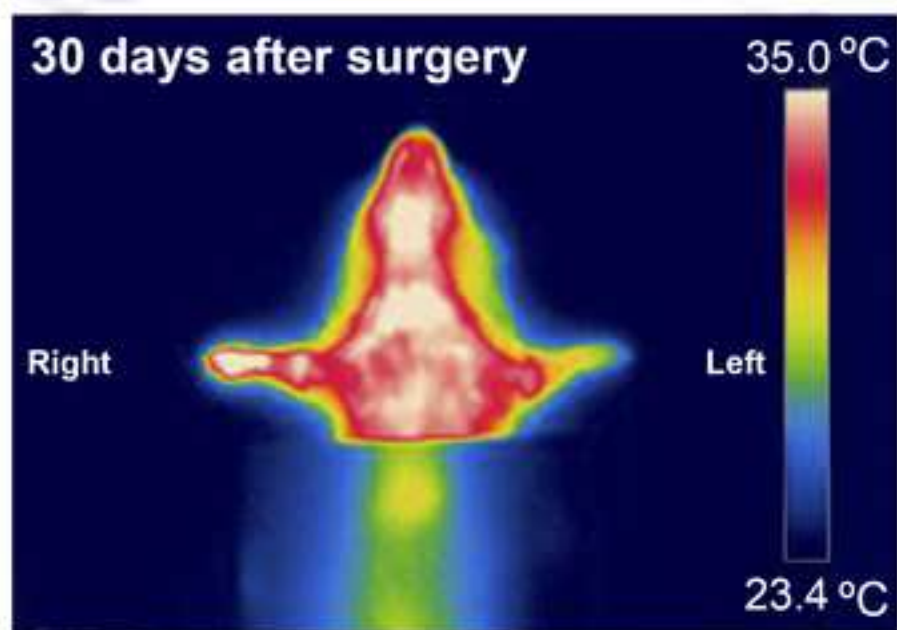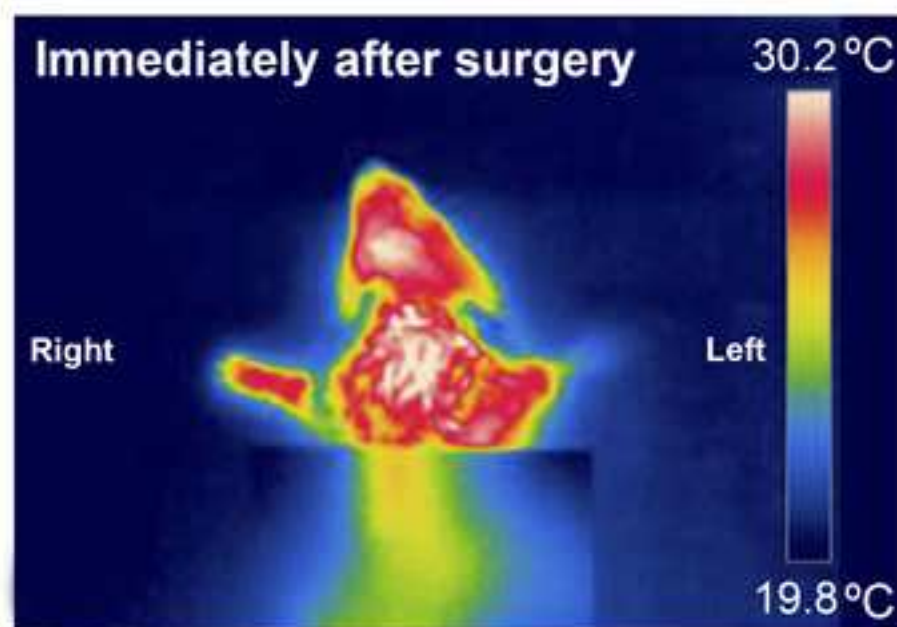
Figure 9

Figure 10

Normal CMAP pattern — Sham Group

Polyphasic CMAP pattern — Nerve graft Group
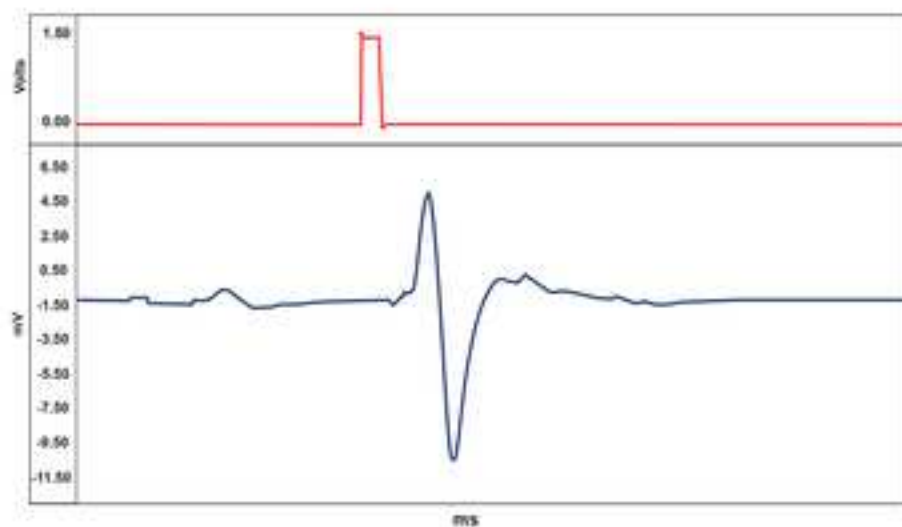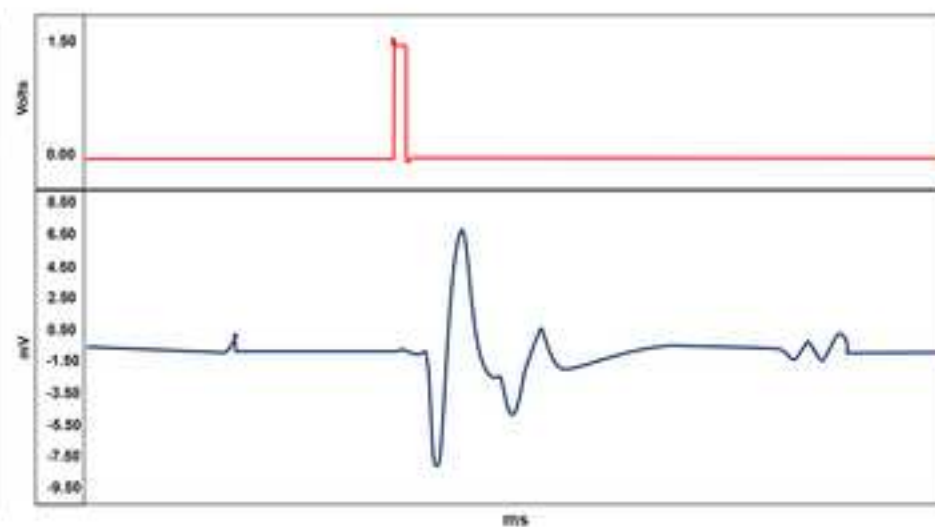
Figure 11

Figure 12

Table 1
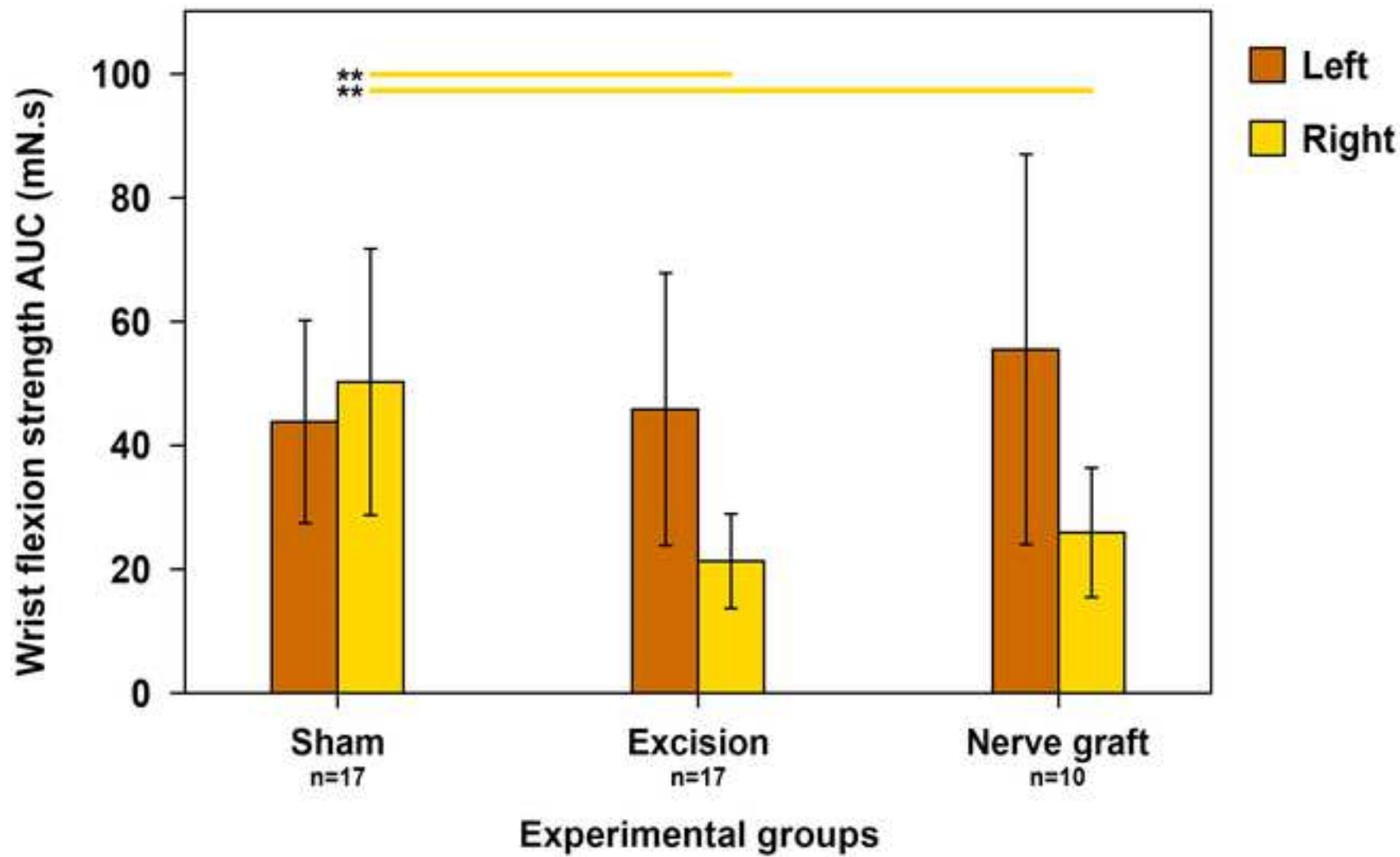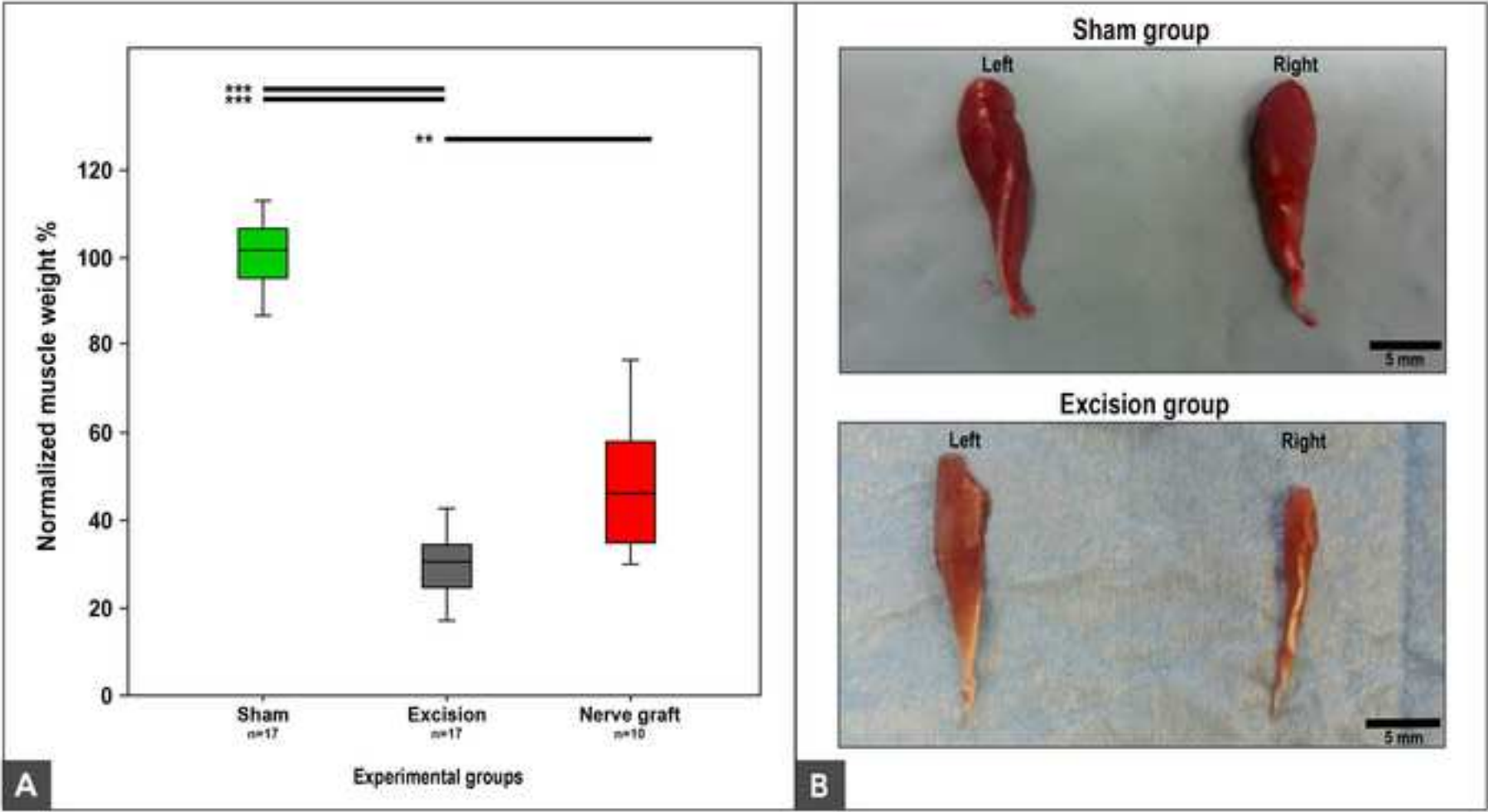
Parameter	Parameter significance	Sham group	Excision group	NG group
Neurological stimulation threshold (%)	Evaluation of nerve regeneration, as there is a minimal number of nerve fibers required to produce either a CMAP or a visible muscle contraction[12]	281.63 ± 271.65	5359.98 ± 3466.52	2108.12 ± 2115.13
Motor stimulation threshold (%)	Evaluation of nerve regeneration, as there is a minimal number of nerve fibers required to produce either a	462.52 ± 118.91	1694.10 ± 503.24	1249.50 ± 503.24
Latency (%)	Assessment of nerve conduction velocity in the fastest nerve fibers, that is to say the largest myelinated fibers[44]	113.55 ± 25.04	N/A	132.80 ± 69.95
Neuromuscular transduction velocity (%)	Assessment of nerve conduction velocity in the fastest nerve fibers, that is to say the largest myelinated fibers[44]	92.01 ± 20.88	N/A	91.30 ± 26.51
CMAPs amplitude (%)	Evaluation of the number of reinnervated motor units[34]	110.63 ±45.66	N/A	41.60 ± 24.84
CMAPs duration (%)	Assessment of synchrony of muscle innervation, which is dependent on the degree of muscle reinnervation and myelination of the innervating motor fibers[44,45]	101.12 ± 23.92	N/A	151.06 ± 54.52

Table 1. Electroneuromyographic assessment
at the end of the experiment

NG, nerve graft

CMAPs, compound muscle action potential.

N/A, non-applicable

All parameters are expressed as percentages of the average contralateral values.

Numeric variables are expressed as average ± standard deviation.

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Acetaminophen	Amazon	
Acland clamps	Fine Science Tools	00398 V
Acland Single Clamps B-1V (Pair)	Fine Science Tools	396
Biogel Surgical Gloves	Medex Supply	30465
BSL Analysis	BIOPAC Systems	
Castroviejo needle holders	Fine Science Tools	12565-14
Clamp applicator	Fine Science Tools	CAF-4
Constante voltage stimulator	BIOPAC Systems	STM200
Cutasept skin disinfectant	Bode Chemie	
Dafilon 10-0		G1118099
Derf Needle Holders 12 cm TC	Fine Science Tools	703DE12
Dry heat sterilizer	Quirumed	2432
Dynamometer	SAUTER	FH5
Electroneuromiography setup	BIOPAC Systems	MP36
Ethilon 5-0		W1618
FLIR Software	FLIR	
Graeffe forceps 0.8 mm tips curved	Fine Science Tools	11052-10
Graph paper	Ambar	
Heat Lamp HL-1	Harvard Apparatus	727562
Heparin Sodium Solution (Heparin LEO 10000IU/ml)	Universal Drugstore	
High-Temperature Cautery	Fine Science Tools	AA03
Homeothermic Blanket System with Flexible Probe	Harvard Apparatus	507220F
Infrared camera	FLIR	E6
Instrapac - Adson Toothed Forceps (Extra Fine)	Fine Science Tools	7973
Iris Scissors 11.5 cm Curves EASY-CUT	Fine Science Tools	EA7613-11
Ketamine hydrochloride/xylazine hydrochloride solution	Sigma- Aldrich	K113
Lacri-lube Eye Ointment 5g	Express Chemist	LAC101F

Mayo Scissors 14 cm Straight Chamfered Blades EASY-CUT	Fine Science Tools	EA7652-14
Meloxicam	Recropharma	Mobic
Methylene Blue solution	Sigma- Aldrich	
Micro Jewellers Forceps 11 cm straight 00108	Fine Science Tools	JF-5
Micro Jewellers Forceps 11cm angulated 00109	Fine Science Tools	JFA-5b
Micro retractor	Fine Science Tools	RS-6540
Micro Scissors Round Handles 15 cm Straight	Fine Science Tools	67
Micro-vessel dilators 11 cm 0.3 mm tips 00124	Fine Science Tools	D-5a.2
Monosyn 5-0		15423BR
Normal saline for irrigation	Hospira, Inc.	0409-6138-22
Operating microscope	Leica Surgical Microsystems	
Skin Skribe Surgical Skin Marker	Moore Medical	31456
Snacks	Versele-Laga	Complete Crock-Berry
Straight mosquito forcep	Fine Science Tools	91308-12
Surgical drapes	Barrier	800430
Veet Sensitive Skin Hair Removal Cream Aloe Vera and Vitamin E 100 ml	Veet	

Comments/Description

https://www.amazon.com/Childrens-Tylenol-grape-flavor-ages/dp/B0051VVVZG

http://www.merciansurgical.com/aclandclamps.pdf

 http://www.merciansurgical.com

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http://www.merciansurgical.com/acland-clamps.pdf

https://www.biopac.com/product/constant-voltage-stimulator-unipolar-pulse/

http://www.productcatalogue.bode-chemie.com/products/skin/cutasept_f.php

http://www.bbraun.com/cps/rde/xchg/bbraun-com/hs.xsl/products.html?prid=PRID00000816

 http://www.merciansurgical.com

http://www.quirumed.com/pt/material-de-esterilizac-o/esterilizadores

https://www.sauter.eu/shop/en/measuring-instruments/force-measurement/FH-S/

https://www.biopac.com/product/biopac-student-lab-basic-systems/

http://www.farlamedical.co.uk/

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https://www.harvardapparatus.com/webapp/wcs/stores/servlet/haisku3_10001_11051_39108_-1_HAI_ProductDetail_N_37610_37611_37613

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http://www.boviemedical.com/products_aaroncauteries_high.asp

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http://www.finescience.de

http://www.merciansurgical.com

http://www.merciansurgical.com

http://www.mcfarlanemedical.com.au/15423BR/SUTURE-MONOSYN-5_or_0-16MM-70CM-(C0023423)-
BOX_or_36/pd.php

http://www.hospira.com/en/search?q=sodium+chloride+irrigation%2C+usp&fq=contentType%3AProducts

http://www.leica-microsystems.com/products/surgical-microscopes/

www.mooremedical.com/index.cfm?/Skin-Skribe-Surgical-Skin-Marker/&PG=CTL&CS=HOM&FN=ProductDetail&PID=1740&spx=1

http://www.versele-laga.com/en/complete/products/complete-crock-berry

http://www.finescience.de

http://www.molnlycke.com/surgical-drapes/

http://www.veet.co.uk/products/creams/creams/veet-hair-removal-cream-sensitive-skin/

Dear Professor Vineeta Bajaj

Editor of the Journal of Visualized Experiments,

We are now resubmitting a revised version of the paper entitled "***Functional and physiological methods of evaluating median nerve regeneration in the rat***" (JoVE59767_R2).
We have performed all the suggested changes to the manuscript and to the video.

We would like to start with by once more thanking you and the referees for the time you have taken to thoroughly review our paper, and for your remarks and suggestions.

We have performed all the changes you suggested, as detailed below:

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

We kept the formatting introduced by the editorial team.

2. Please address all the specific comments marked in the manuscript.

We performed all the changes suggested by the reviewers throughout the article.

3. Please reword 376-378, 380-381, 388-389, 438-439, 520-521, 642-645, 649-651, 682-685, 694-695 as it matches with previously published literature.

We rephrased the mentioned sections of the text. These changes have been highlighted in the manuscript using the "track changes mode".

4. Figure legends for Figure 4,5,6,7 matches with previously published literature. If the figures are reprinted, please include reprint permission. If not then please reword.

These figures are original and have not been published elsewhere. We reworded the mentioned figure legends.

5. Please ensure that the protocol length is no more than 10 pages including headings and spacings.

Presently, the protocol is composed of 9 pages (pages 3 to 11 of the manuscript).

Video:

1. 0:10 – Please remove the titles (MD, PhD) from the name. Also, do not include plastic and reconstructive surgeon, instead please include the hospital/institute affiliation.
2. 2:49 – When showing the name of the talent, please remove MSci, and Biomedical Engineering. Please include the name of the hospital, university, etc (e.g., NOVA Medical School).
3. 0:46 – Please change the subheading to match the section title in the protocol text.
4. 2:04 – Please remove the subheading "experimental groups" as this is not present in the text. Please make this section continuous.
5. Please have the same subheadings both in the text and the video. Please remove 3.1Median Nerve exposure from 0.46 and change it to Median nerve surgery as in the text.
6. Please remove the numbering from the subtitle cards throughout the video and from the representative result and conclusion title card as well.
7. 6:01: The VO is cut at this part.

We have performed all the requested changes to the video.

Once more, we believe that the modifications we have performed in the video and manuscript according to the reviewers' specifications have significantly improved the original paper.

As we noted earlier, arguably, using the median nerve (**MN**) of the rat, it is possible to adequately replicate peripheral nerve lesions in humans.[1-3] In this paper **we present a model of peripheral nerve lesion and repair in the rat**. In particular, we demonstrate in a detailed fashion the steps required to create and repair different types of MN lesions in the rat. Moreover, we introduce different settings used to simulate postoperative physiotherapy. Finally, we illustrate how to evaluate functional recovery of this nerve using several tests.

We strongly believe that **this model is particularly indicated to replicate the clinical scenario, facilitating extrapolation of results to the human species.**

We base this description in our groups' 12-year experience with the use of this model for both teaching and research purposes.[1,4] To make this subject more perceptible to a wider audience, we have included an original professional-drawn diagram illustrating the relevant anatomy of the MN in the rat (**Figure 1**).

Hence, we believe this paper will undoubtedly be of great interest to the readers of ***JoVE***, as the MN model has multiple advantages relatively to the most commonly used sciatic nerve model.[1,5-7]

We very much hope you decide to publish it!

If you have any doubts or queries, please do not hesitate to contact us.

Thank you so much for your time and kind attention.

The authors

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1   Casal, D. *et al.* Reconstruction of a 10-mm-long median nerve gap in an ischemic environment using autologous conduits with different patterns of blood supply: A comparative study in the rat. *PLoS One.* **13** (4), e0195692, doi:10.1371/journal.pone.0195692, (2018).

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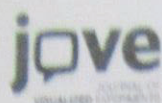
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Author(s): *Diogo Casal, Eduarda Mota-Silva, Inês Iria, Diogo Pais, Ana Ferreira, Sara Alves, Cláudia Pen, Luís Mascarenhas-Lemos, Ralf Ferreira Silva, Mario Ferraz Oliveira, Valentina Vassilenko, Paula Videira, João Goyri-O'Neill*

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