Critical analysis of the value of the rabbit median nerve model for biomedical research on peripheral nerve grafts.

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ABSTRACT

The rabbit has been proposed to represent an animal model that allows studying peripheral nerve regeneration across extended gap lengths. We describe here our experiences with the rabbit median nerve model and the obstacles it comes along with. This short communication is meant to inform the community and to prevent other researcher from investing time and animal lives in a model with low translational power.

MAIN TEXT

Novel bioartificial or tissue engineered nerve guidance channels could serve as substitute or alternative to autologous nerve grafts for peripheral nerve repair. New developments are pre-clinically studied in animal models with different potentials for translation into regenerative medicine.

The rat sciatic nerve model is the most commonly used pre-clinical animal model (Angius et al., 2012; Geuna, 2015). The model is economic, it is easy to compare new results to the literature and, most importantly from the translational point of view, the model allows studying the outcome of functional peripheral nerve recovery (Navarro, 2016). Over the last two decades, also the use of the rat median nerve model has been increased in pre-clinical research on bioartificial nerve grafts and the rabbit has been proposed as animal model that allows investigations on extended gap lengths up to 3-6cm in the median nerve (Ronchi et al., 2019)

We have broad experience in using rat and mouse models for evaluating peripheral nerve degeneration and regeneration and describe below the anatomy of the rabbit median nerve, and give a report on our own experiences with this model. We define several obstacles of the rabbit median nerve model that are considerably reducing its value for pre-clinical nerve graft evaluation.

On the first look the model appears an optimal one because the rabbit brachial plexus demonstrates a high similarity to the human brachial plexus (Reichert et al., 2015). From the available literature (Mencalha, Sousa, Costa, & Abidu-Figueiredo, 2016; Reichert et al., 2015; Reichert et al., 2014, Popesko, Rajtová, & Horák, 1992) it can be summarized that the median nerve separates from the ventral rami of the cervical C7, C8, the thoracic Th1, and in rare cases the Th2 spinal nerves.

At their origin the median and ulnar nerve form a common trunk and only separate in the area where the axillary artery and vein gives rise to the median artery. This artery

travels in between the median and the ulnar nerve from their bifurcation point towards the medial elbow (Popesko et al., 1992). The median nerve is located cranial to the median artery and the ulnar nerve caudal to it. From the elbow, the median nerve runs towards the cranial lower limb along the radial bone passing by the cranial rim of the pronator teres muscle, while the ulnar nerve travels more caudally along the ulnar bone (Popesko et al., 1992). On its way towards the lower limb, the median nerve gives immediately rise to a muscle branch innervating the pronator teres muscle. More distally it innervates also the flexor muscles of the paw. **Figure 1** is illustrating the anatomic situation as it presented during our own examinations.

For nerve surgery, general anaesthesia was induced by intraperitoneal application of ketamine (25mg/kg body weight) and xylazine (3mg/kg bodyweight) and the animals were placed on their back with both forelimbs tape-fixed (left forelimb in a 45° angle to the trunk and with its lateral surface fixed down on the surgery plate). On the left forelimb, a skin incision was made with a surgical blade along the humerus. The subcutaneous fat tissue was pushed aside by blunt dissection thus exposing the median nerve. The wound was kept open with the aid of small retractors.

The median nerve was exposed along its way from axilla to elbow and 2-5 mm more into distal direction. It could then be securely transected with a single microscissors' cut using the neighbouring olecranon as a landmark (**Figure 2**A, B). Sterile scale paper was used to exactly measure the gap length created by transection and the length and location of the implanted nerve graft (**Figure 2**B).

For nerve autotransplantation, a 2.6 cm median nerve segment was excised from the distal transection site into proximal direction. Therefore the nerve was separated from the distal muscular branch of the musculocutaneous nerve. Before re-sutured, the nerve segment was reversed (distal – proximal). Two epineurial sutures (10-0 or 9-0) were placed 180° apart, each at the proximal and distal coaptation sites. Each suture was placed by passing first the needle through the proximal nerve end (out-in, exit underneath the epi-perineurium at the cross-section of the nerve fascicle), then through the epi-perineurium of the distal nerve end (in-out), and then knotted for loosely bringing the two nerve ends together (**Figure 2**C).

For nerve conduit repair, 2 cm of the proximal median nerve end have been removed prior to implantation of a 3 cm transparent chitosan guide. The nerve guide was sutured in an out-in/in-out fashion with epineurial sutures. The sutures pulled the nerve ends straight into the lumen of the conduit and exact tube-site placement of the

sutures ensured production 2.6 cm gap defects between the nerve ends. The proximal nerve end was sutured with exclusion of the distal muscular branch of the musculocutaneous nerve. After both nerve ends had been sutured, sterile NaCl solution was injected into the nerve conduit (e.g. using a 27Gx1/2'' needle) to avoid blood clot formation inside the hollow lumen of the nerve guide (**Figure 2**D).

After nerve reconstruction was finished, the area was flushed with cold saline solution. Finally, the repaired nerve was covered with the fat tissue flap placed aside at the beginning of the surgery, and the skin was closed with 4-0 to 3-0 sutures. The wound was disinfected and covered with collagen spray.

When the animal was still anesthetized, an Elizabethan collar was put to prevent events of immediate licking and suture removal for up to 7-10 days after surgery. During this time the animals and their wounds were visited 2-3 times daily.

It has to be considered that the anatomy of the rabbits' paw does provide thenar muscles to analyse palmar muscle reinnervation by electrodiagnostical recordings. We therefore decided to record evoked compound muscle action potentials (CMAPs) from the lower forelimb. Specifically, the flexor carpi radialis muscle was selected because it is located distal to the olecranon and innervated by a muscular branch of the median nerve. We initially planned to do serial non-invasive electrodiagnostic recordings in order to depict the progress of reinnervation and functional recovery. But our transcutaneous recordings from the flexor carpis radialis muscle in the lower forelimb of the rabbits, turned out to be vulnerable to co-stimulation of neighbouring muscles. The conclusion was drawn from the fact that although clearly depicting a dramatic difference in the calculated mean amplitude heights between the reconstructed and the healthy contralateral side, transcutaneous recordings always displayed evocable CMAP signals. And this was also the case when the following exploration of an implanted nerve conduit revealed that the nerve did not regenerate through the same. The transcutaneous approach had therefore to be judged as biased (high risk for false positivity). Thus we replaced the repetitive evaluation approach by end-point analysis with direct nerve stimulation and recording from the exposed muscle at the end of the observation time.

For end-point CMAP recording, the animal was anaesthetised (as above) and placed in the same position as for the surgery on a heating pat to stabilize the body temperature.

A ground needle electrode was placed subcutaneously at the lateral side of the trunk of the animal. The flexor carpi radialis muscle was exposed as was the muscular branch of the median nerve innervating the same muscle and a monopolar needle electrode was inserted into the belly of the muscle. The reference needle electrode was placed into the tip of one digit of the same paw. The median nerve was stimulated with single electrical pulses (100 µs duration and supramaximal intensity) delivered by a steel hook electrode proximal to the injury. Co-stimulation of the ulnar nerve was avoided by shielding it with plastic pads.

The CMAPs were recorded and displayed in the oscilloscope of an EMG machine (Dantec Keypoint Portable System, Natus Europe). Electrical stimuli of 100 µs duration were delivered with progressively increasing intensity (starting with 1 mA and increasing in increments of about 2 mA) until a maximal amplitude CMAP is obtained, that does not increase with further stimulation (until about 30% over the stimulation that elicits the maximal CMAP). The filters for recording were set at: 2 Hz and 2 kHz. Control values were recorded from the right unoperated forelimb.

When analysing the amplitude height of the recorded CMAPs upon stimulation of the reconstructed and the healthy contralateral nerves, clear differences should be detectable depending on the success rate of nerve regeneration.

Representative example recordings from an animal of the autograft group revealed a mean amplitude height of 35.86 mV (±0.52 standard deviation) on the contralateral healthy side. On the reconstructed side the values were still dramatically reduced 4 months after surgery (10.34 ± 0.37 mV). This finding was unexpected given the commonly good results reported from autograft groups in rats and mice.

Overall, from our attempt to use the rabbit median nerve as a challenging gap length model for nerve graft evaluation, we had to conclude that the model has serious limitations. (1) Although we did not observe automutilation behaviour affecting the paws, we still observed loosening of the skin sutures, whenever the animals had access to the suture site for more than a few minutes. The animals started to clean themselves and to remove the sutures. (2) Furthermore, we observed disconnection of the nerve guides from the epineurial sutures that had been applied during surgery. Since this was never observed in our previous rat or mouse studies, this had to be attributed to the fact that the movement of the rabbit puts too much sudden tension on the median nerve when reconstructed with a nerve guide. For autograft repair we did not observe a disconnection of the nerve sutures. (3) It has to be considered that

functional evaluation, by means of electrodiagnostic recordings, could only be performed as end-point measurements. The anatomy of the rabbit forelimb does not allow for reliable transcutaneous stimulation of the median nerve alone and co-stimulation of the neighbouring ulnar nerve gives serious bias to the results. (4) Repetitive anaesthesia is also an issue, in our hands the rabbits showed some habituation effect after the initial anaesthesia, resulting in the need to increase dosage and reapplication of anaesthetics for consecutive anaesthesia, sometimes even above the tolerated maximum.

In conclusion, we would not recommend using the rabbit as an animal model for median nerve injury and repair in biomedical research. We hope that the readership finds our case study helpful in order to avoid wasting time and animal lives with a model of low translational power.

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Ethics statement:

All procedures were approved by the Bioethical Committee of the University of Torino and by the Italian Ministry of Health. Moreover, these procedures agree with the National Institutes of Health guidelines, the Italian Law for Care and Use of Experimental Animals (DL26/14), and the European Communities Council Directive (2010/63/EU).

Conflict of interest statement:

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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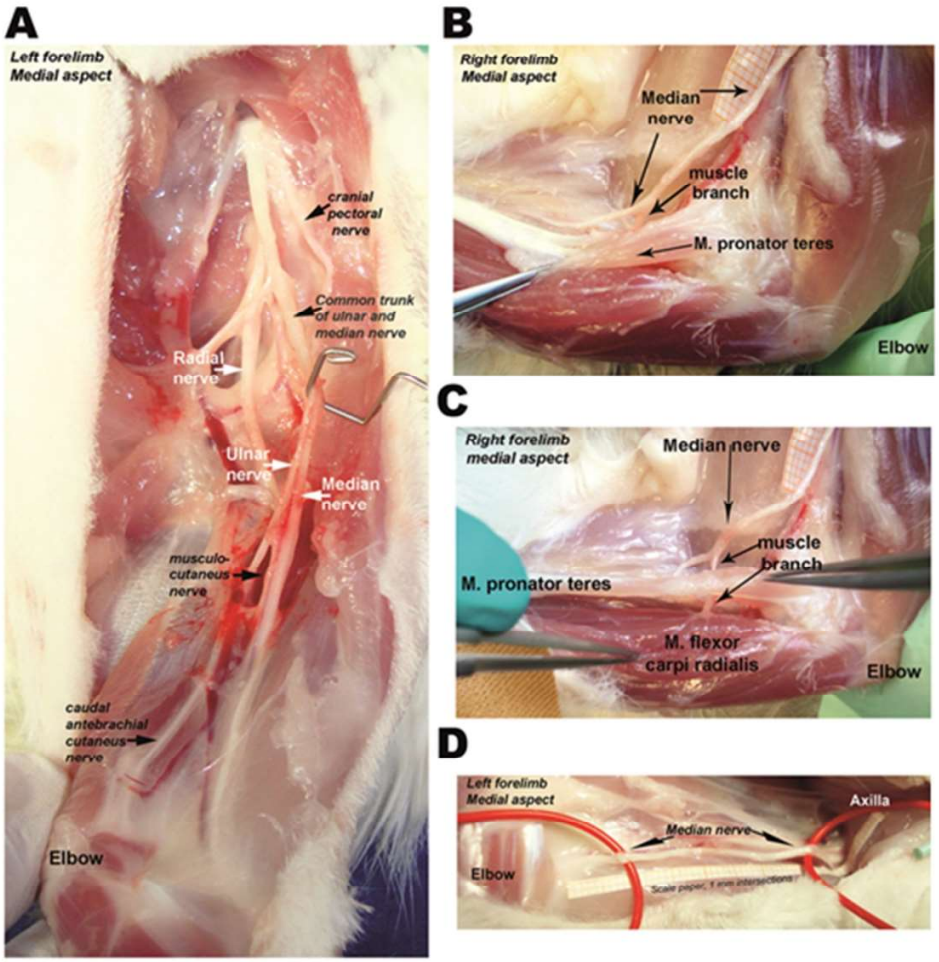
Figure legends

Figure 1: (A) Medial aspect of the left forelimb: after dissection of the cutaneous trunci muscle and the pectoral muscles, the course of the median nerve can be followed from its bifurcation with the ulnar nerve down to the cranio-medial elbow. (B) + (C) Medial aspect of the right forelimb: after the medina nerve passed the elbow, its first muscle branch exits to innervate the flexor carpi radialis muscle. This muscle branch travels below the pronator teres muscle. (D) Medial aspect of the left forelimb: the median nerve can easily be deliberated along a distance of 2.5 – 3 cm in order to apply nerve transection injury and long nerve gap repair.

Figure 2: (A) Illustration of the surgical design for nerve transection and (B) a representative image of the nerve preparation for transection. Along the nerve in (B) a scale paper has been placed indicating a distance of 4 cm between axillary area (upper end, right) and the olecranon (lower end, left). (C) + (D) Representative images of a rabbit median nerve autograft (C) and a nerve conduit (chitosan nerve guide in D) bridging a 2.6 cm gap in the rabbit median nerve. Along the nerve a scale paper has been placed indicating a distance of 4 cm between axillary area (upper end, right) and the olecranon (lower end, left). White arrows point to the distal muscular branch of the musculocutaneal nerve. The ulnar nerves are covered by a strip of scale paper indicating the 2.6 cm length of the gap or distance between the proximal and distal sutures, respectively.



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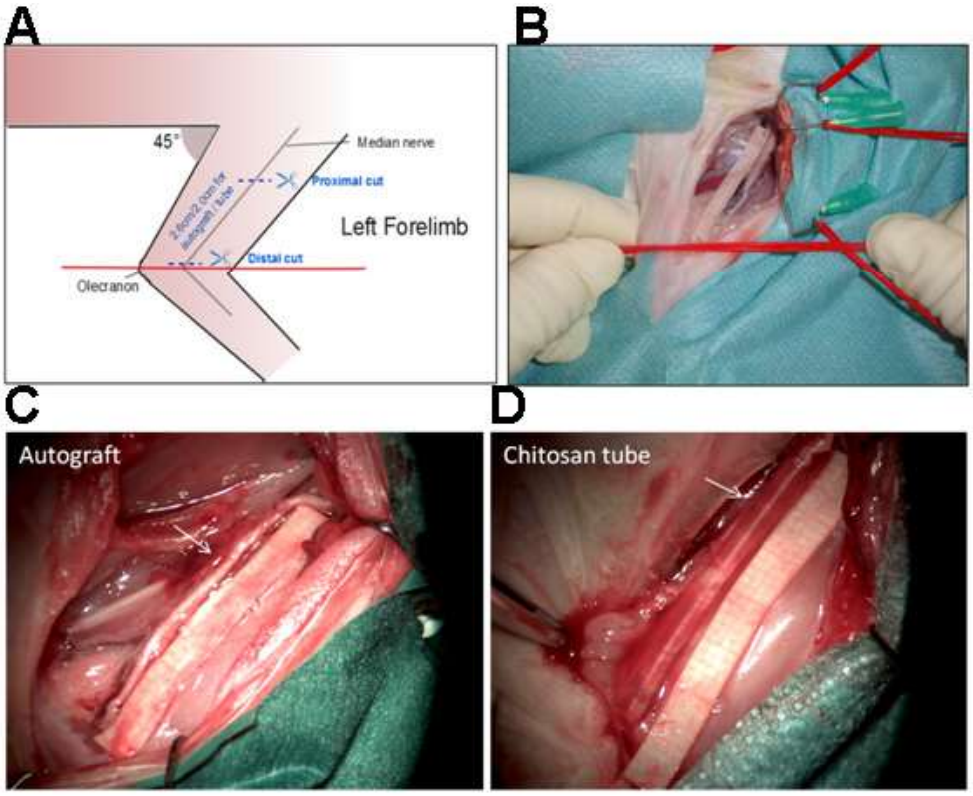

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