Ph.D. Thesis

In vivo epineural nerve staining with methylene blue

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1. Introduction

Dupuytren's contracture is a progressive fibroproliferative disease of the hand where the thickening of the palmar and digital fascia causes flexion contractures of fingers which can result in compromised hand function and quality of life. Most frequently it is an autosomal dominant disease with variable penetrance. Many nongenetic causes may also play a role in the onset and severity of the disease, the prevalence can approach 30%. Recent genome-wide association studies identified causal genes which are responsible for the imbalance of WNT signaling pathway, abnormal extracellular matrix modulation and inflammation that play a key role in the development of fibromatosis in Dupuytren's disease.

Despite the increase of non-surgical treatment of Dupuytren's contracture, limited fasciectomy is still the gold-standard treatment. The rate of iatrogenic nerve injury in limited fasciectomy surgery can reach 3.4%. Nerve injury occurs more commonly in case of dislocated digital nerves by the Dupuytren's spiral cords and in revision surgeries. The challenge during fasciectomy is to identify and protect the digital nerves which can have an altered anatomical position usually caused by the pathologic spiral cord passing around the nerve drawing it superficially and toward the midline of the finger. About 20-30% of patients undergoing limited fasciectomy can expect recurrence. The risk of digital nerve injury is greater during revision surgery caused by the combination of scar tissue from the previous surgical intervention, the recurrent Dupuytren's tissue, and the changed anatomical position of nerves.

A simple nerve staining technique could aid intraoperative location and protection of the digital nerve by improving its visibility and facilitating separation of the nerve from the Dupuytren's tissue.

We chose methylene blue (MB) for our study as it is a commonly used stain with a wide range of therapeutic applications. To the best of our knowledge, the efficacy and safety of the direct epineural MB injection has never been tested.

2. Aims

The challenge during fasciectomy is to identify and protect the digital nerves which can have an altered anatomical position. A simple nerve staining technique could aid intraoperative location and separation of the digital nerves from the Dupuytren's tissue, and the risk of iatrogenic nerve injury could be reduced.

The following aims were set in this study:

1. To design a simple *in vivo* nerve staining technique which can be used intraoperatively to mark peripheral nerves. Our theory was that epineural injection and administration of methylene blue into a peripheral nerve does not cause damage to the nerve, and it provides an improved visibility of the nerve, facilitating its dissection.
2. To evaluate the nerve staining potential of methylene blue on animal model. Animal experiments were designed to study the *in vivo* nerve staining potential of methylene blue. We supposed that diluted methylene blue is a suitable agent to mark peripheral nerves *in vivo*.
3. To determine the length of the nerve segment stained with diluted methylene blue. We supposed that methylene blue gives macroscopically well-recognizable blue coloring in the nerves *in vivo*, which could significantly improve the surgeon's ability to locate and preserve the nerves in Dupuytren's surgery.
4. To assess the safety of injecting diluted methylene blue into a peripheral nerve and whether this causes structural or functional damage to the nerve. Methylene blue is a commonly used stain with a wide range of therapeutic applications. We anticipated that methylene blue will not cause functional and structural damage to the stained nerve.
5. Finally, methylene blue has been injected into cadaveric human nerves to assess whether the nerve staining method could be a suitable technique to mark human digital nerves.

3. Materials and Methods

3.1. Animals

Sciatic nerves of twelve-weeks-old male Wistar rats were used as model because its diameter (1-1.5 mm) is similar to the human digital nerves. Animals were housed in temperature and humidity controlled 12 h light-dark cycle environment (lights on at 6 am) in standard polycarbonate cages (400 mm × 250 mm × 200 mm) in two rats per cage groups at the animal facility of the Department of Anatomy, University of Pécs. Rats were provided *ad libitum* with standard rodent chow and drinking water. *In vivo* experimental procedures were permitted by the National Food Chain Safety Office in Hungary (permission No: BA02/2000-42/2018). The licenses were given based on the scientific approvals of the Animal Welfare Committee at University of Pécs and the National Scientific Ethical Committee on Animal Experimentation in Hungary.

3.2. Experimental design

In order to test the safety and efficacy of epineural injections three experimental steps were designed using the rat sciatic nerve as model. Human cadaveric experiments were also performed to test the efficacy of the technique on human digital nerves.

3.2.1. Experiment No. 1

In order to test whether the nerve injection is safe, the rats were divided into three groups. In group one (n=5) the sciatic nerves were visualized and prepared (sham surgery). In group two (n=5) the nerves were visualized and needle (29G injection cannula) insertion was performed. In group three (n=5) after preparation and needle insertion, 40 µl of saline solution was injected. The nerve function was assessed by dynamic plantar aesthesiometer (DPA) and morphological tools (histology) were applied to assess the structural effects of these interventions. Any signs of motor deficits of sciatic nerves were also observed.

3.2.2. Experiment No. 2

Six sciatic nerves were injected with 40 µl 1m/m% stock methylene blue solution to determine the histoanatomical localization of the injected dye in the tissue.

In order to determine the optimal minimal concentration of methylene blue solution that provides a well-recognizable blue color besides the 1% stock solution, 1:40, 1:80 and 1:160 physiological saline dilutions of the 1% solution were also pre-tested on two nerves, respectively. The test of a series of increasing dilutions revealed that 40 µl of the 1:80 diluted 1% stock solution of methylene blue gives macroscopically well-recognizable blue coloring in the nerves in vivo.

3.2.3. Experiment No. 3

In order to test the effects of nerve staining, both sciatic nerves were prepared in six rats. The left nerve was injected with 40 µl saline while the right one was treated with 40 µl of 1:80 MB. We measured the length of the stained nerve segments. The primary goal was here to see the functional effect of the treatment by DPA measurement on the 7th and 10th postoperative days. The motor function of sciatic nerves was also observed. The secondary endpoint was to test if the sciatic nerves were damaged histologically. Therefore, on postoperative day 11, rats were euthanized and the sciatic nerves were collected for histology.

3.2.4. Experiments on human cadaver hands

Human cadaver hands were used to test the efficacy of nerve injection at the Department of Anatomy, Medical School, University of Pécs. The use of human body for testing operation techniques was approved by the Ethical Commission for Research at Pécs University (Approval No: PTE KK RIKEB No.: 6466, 43/2017).

In the first experiment after dissection of the common and proper digital nerves on formalin fixed cadaver hand, four digital nerves were injected with 40 µl of 1:80 diluted 1% methylene

blue solution. The spread of methylene blue and the length of the stained nerve's segments were measured.

In the second experiment we dissected four digital nerves on a cadaver hand which previously did not undergo fixation. Six digital nerves were stained at the level of distal margin of palmar aponeurosis. In this experiment we increased the quantity of the injected dye to 200 µl on two nerves in order to increase the length of the stained segment. We measured the spreading potential of MB and we also measured the lengths of stained nerve segments.

3.3. Surgery

In all experiments, animals were anaesthetized with intraperitoneally injected ketamine (78mg/kg) and xylazine (13mg/kg). In Experiment 1, for sham surgery, the sciatic nerves were approached and dissected. For needle insertion control, a 29-gauge cannula was introduced into the epineural space. For the volume injection control, 40 µl of saline was injected into the epineurium. In Experiment 2, 40 µl of MB stock solution, and 40 µl saline dilutions (1:40, 1:80 and 1:160) were injected into the epineurium. In Experiment 3, nerves were injected either with 40 µl physiological saline or they were administered with 40 µl of 1:80 diluted MB solution. Solutions were pre-filled into polyethylene tubes with 29-gauge cannula connected to a Hamilton syringe. Sciatic nerves were stained distally to the puncture point. The wounds were closed with 4.0 absorbable suture.

The animals' health condition and wound healing, moreover the general behavioral examinations for neuropathy according to Seltzer were observed on all postoperative days. Rats were individually placed on a table where they were allowed to explore. The observer studied if the rats used their hind limbs symmetrically. In case of a sciatic nerve injury rats obviously save their affected hind limb, and if a muscle palsy also occurs, the foot turns into a strongly pronated position that is associated with limping. This was examined by lifting the rat holding on the tail in a way that the forepaws stayed on the table. The observer recorded if any signs of nerve injury was visible.

3.4. Measurement of mechanonociceptive threshold

Dynamic plantar aesthesiometer (DPA, Ugo Basile 37000, Comerio, Italy) was used to measure mechanical hyperalgesia. After two pre-surgery control measurements, animals were re-tested on the 7th and 10th postoperative days. Rats were placed into an observation chamber on a mesh platform. The hind paw mid-plantar surface was tested by a straight metal filament lifting with increasing upward force till paw withdrawal. Stop signal was attained when the animal removed the paw or when the cut-off force of 50 g was reached, but latest after 10 seconds. Mean pain threshold values were calculated from the average of three tests.

3.5. Euthanasia, tissue collection

Rats were injected with an overdose of urethane (2.4 g/kg), intraperitoneally. Animals were transcardially perfused with ice-cold 0.1M phosphate buffered saline (PBS, pH: 7.4) followed by 4% paraformaldehyde solution in Millonig buffer (pH 7.4). The sciatic nerves from each animal were dissected and removed for microscopic examinations.

3.6. Microscopy, imaging and morphometry

Four HE stained sections of each nerves were photographed using a Nikon Microphot FXA microscope with a RT camera (Nikon, Tokyo, Japan). Nerve fiber quantitation was performed using images obtained with 40x objective lens (Nikon ApoPlan 40). The manual nerve fiber counting was performed using the multipoint tool of the Image J software (version 1.42., NIH, Bethesda, MD). Nerve fiber per pixel values were averaged using the data of four sections, and this value represented one nerve in the statistics.

3.7. Statistics

All data were presented as the average of the group ± the standard error of the mean (SEM). All datasets were tested for homogeneity of variance (Bartlett's chi-square test) and normal

distribution (Shapiro-Wilk test). The mechanical pain threshold values in Experiment 1 were tested at each time point using one-way analysis of variance (ANOVA) where the categorical predictor was the 'treatment'. Student's t test for independent samples was used to assess the mechanical pain threshold values at all time points and also for the nerve fiber density values, obtained in Experiment 3. Alpha value was set to 5% in all cases.

4. Results

4.1. Experiment No. 1

Effects of sham surgery, needle insertion and saline injection on sciatic nerve function were compared. Unaltered nerve morphology was found in all groups.

DPA measurements revealed no differences across groups in the first (ANOVA $F_{2,12}$=0.65 p=0.53) and second ($F_{2,12}$=0.93 p=0.42) preoperative tests. No statistical differences developed in mechanical pain threshold values on 7th ($F_{2,12}$=0.40 p=0.69) and 10th ($F_{2,12}$=3.40 p=0.06) postoperative days. No muscle palsy or wound healing problems were observed.

4.2. Experiment No. 2

Injection of 40 μl 1% MB resulted in an immediate strong blue coloring of the sciatic nerve. In order to determine which neural histological compartment the dye spread along, native and hematoxylin-eosin stained consecutive serial sections were examined. Thick (50μm), native preparations were suitable to visualize the presence of the methylene blue dye in the sciatic nerve. Comparisons with adjacent HE stained sections allowed us to determine that the methylene blue dye accumulated in the epineural compartment of the nerve. We did not detect blue color inside the fascicles suggesting that the perineurium remained intact.

During the surgery, we realized that even a very small droplet of the stock solution spread in the wound outside the nerve was able to stain all the adjacent tissues blue, resulting in a considerable blue "background" decoloring of the operation area. To select the lowest

concentration of methylene blue solution still providing a proper labelling with no significant "background" staining a series of dilutions have been injected to different animals.

These results suggest that injection of 1:80 dilution provides the optimal balance between a visible blue color within the epineural space for a considerable distance and preserving the endoneural components.

4.3. Experiment No. 3

Saline and methylene blue injected sciatic nerves were compared in the same rats. The injection of the 40 µl volume of 1:80 diluted methylene blue solution allowed us to stain a 18.18 mm (range 10.00 mm-30.10 mm) length of the nerve distal to the injection site.

DPA measurements revealed that the saline and methylene blue-treated limbs showed the same mechanical pain threshold values in the first (Student's t test: p=0.34) and second control tests (Student's t test: p=0.34). The comparison on the 7^{th} (Student's t test: p=0.68) and 10^{th} (Student's t test: p=0.21) postoperative days also revealed no significant difference in the pain threshold values on the plantar surface of the hind limbs. The regular observation of the rats after the surgery revealed that their gait remained normal. None of the rats developed an abnormal foot posture or contractures indicating the loss of motor control in some muscles. Examination of hematoxylin-eosin stained preparations revealed no recognizable histological changes in the nerves upon methylene blue injection in comparison with the saline injected control nerve on the contralateral side. This observation was further supported by the quantitation of the nerve fiber density in a 40x magnification objective lens field where no difference was found between experimental groups (Student's t test: p=0.91).

4.4. Digital nerve staining on human cadaver hands

In order to test the possible human application of methylene blue nerve staining technique, experiments were performed on human cadaver hands.

In our first experiment, human cadaveric digital nerves fixed with formalin were examined. By using 40 µls of 1:80 diluted methylene blue solution, we were unable to stain nerves previously fixed with formalin. This finding may be explained by well-known effect of fixation resulting in considerable shrinkage of the tissue. Most likely, this fixation-related shrinkage limited the epi- and perineural spaces and resulted in unsuccessful staining.

In our second experiment on previously non-fixed human cadaver digital nerves, the injection of 40 µls 1:80 diluted methylene blue solution stained on average a neural length of 13mm +/- 1.5 mm. The polyethylene tubes attached to the Hamilton syringe did not allow to increase the pressure of the injected solution. Therefore, we increased the volume of the injected dye to 200 µl in order to stain a longer segment on two nerves. Whilst the length of the stained segments increased, it was not proportional to the larger volume of the methylene blue solution. Nevertheless, the average length of the stained nerve reached 18 mm.

5. Discussion

In our study we put forward the hypothesis that the epineural methylene blue injection is a safe method to mark nerves during surgeries. Our idea was that the method may help to solve the technical challenge of separating the digital nerves from pathologic Dupuytren's cord and from the scar tissue during revision Dupuytren's surgeries. Our above described results support our hypothesis as discussed below.

First, we tested the effect of nerve preparation, epineural needle insertion, and saline injection on neural structure and function in a rat model revealing that none of the procedures affected nerve function, as mechanical pain threshold remained at the baseline level and motor function remained intact. The DPA measurement is a well-trusted, widely applied test, used to assess neuropathy-related reduction of mechanical pain threshold. Unaltered histomorphology was in full agreement with intact nerve function. The absence of muscle palsy-related foot pronation upon methylene-blue injection indicates preserved motor function.

In order to determine which connective tissue compartment of the nerve allows the diffusion of the dye, the stock solution was injected in Experiment 2. The color of methylene blue was observed exclusively in the epineural connective tissue space using native and HE stained consecutive serial sections. In contrast, no diffusion of the dye was observed inside the

fascicles in the endoneural space. This suggests that the barrier of the epithelial part of the perineurium remained intact. This is supported by the fact that the nerve fibers did not suffer functional and morphological damage.

During the surgery, we realized that even a very small droplet of the dye spread in the wound outside the nerve was able to stain all the adjacent tissues blue, resulting in a considerable blue "background" decoloring of the operation area. This observation, and the very intense blue color suggested us to reduce the concentration of the dye. After testing multiple dilutions, we decided to choose the 1:80 dilution of the 1% stock solution. The treatment with this concentration caused an obvious blue color in the nerve that helped its identification in the surrounding tissue, but it did not cause any obvious decoloring in the wound if some limited but unwanted leakage occurred at the time of injection or right after the needle removal.

Finally, the effect of 1:80 diluted methylene blue was compared with the effect of intraneural saline injected into the contralateral sciatic nerve of the same rat in Experiment No. 3. In this setup, consistently with the findings in Experiment No. 1, the mechanical pain threshold values remained normal, and no significant sensory or motor deficit has been occurred in the limbs. The absolute pain threshold values were in all cases above 46 grams. In neuropathy models, DPA values above 40 grams are considered as control values. In full agreement with these data, no sign of morphological damage was recorded in the HE-stained sections of the treated nerves. In addition, the nerve fiber density revealed no change upon dye injection.

In our animal model, 40 µl of the 1:80 methylene-blue labelled a nerve segment of 18 mm which distance would be useful during primary and revision Dupuytren's surgery. The methylene-blue might be injected right proximal to the segment that is hidden in the Dupuytren's tissue, the stained nerve segment could aid intraoperative location of the digital nerve by improving it's visibility and facilitating separation of the nerve from the Dupuytren's tissue. If necessary, further stepwise dye injection might be possible. However, the spreading of epineural methylene-blue injection in the Dupuytren's surgery remains to be tested as well as the potential leakage through the proximal puncture points.

We have demonstrated here that the injection of diluted methylene blue into the epineurium of the nerve is an effective and safe nerve staining method which has no negative effect on sensory and motor function of the rat sciatic nerve.

In order to test the possible human application of methylene blue nerve staining technique, experiments were performed on human cadaver hands.

In our first experiment we were unable to stain human digital nerves previously fixed with formalin. The damage of epi- and perineural spaces can be responsible for the unsuccessful results.

In our second experiment on previously non-fixed human cadaver digital nerves, the injection of 40 µls 1:80 diluted methylene blue solution stained on average a neural length of 13 mm. The polyethylene tubes attached to the Hamilton syringe did not allow to increase the pressure of the injected solution. Therefore, we increased the quantity of the injected dye to 200 µl in order to stain a longer segment of the nerve. Whilst the length of the stained segments increased, it was not proportional to the larger volume of the methylene blue solution. The average length of the stained nerve was 18 mm.

Further tests on human cadaveric hands are planned to examine how the methylene-blue spreads in normal and in potentially compressed and displaced digital nerves in Dupuytren's disease. Further experiments are required to test if a second injection distal to the already stained segment will efficiently elongate the marked distance in cases of longer segment trapped in the scar tissue. Subsequent to ethical approval, a preliminary surgical trial on patients with Dupuytren's contracture may also be performed based on our encouraging results.

We conclude that the epineural injection of 1:80 diluted methylene blue is an effective and safe *in vivo* nerve staining method. We have demonstrated that diluted methylene blue injected into the epineurium of the nerve has no negative effect on sensory and motor function of the rat sciatic nerve. This technique could ease to locate and visualise the nerve during surgery. In full agreement with this, our tests on human cadavers revealed that human digital nerves may be marked by these techniques also. We are also aiming to test this technique on cadaver's hand affected with Dupuytren's disease. Ultimately, future clinical human intraoperative tests are required to study the efficacy of the *in vivo* methylene blue nerve staining technique.

6. Conclusions of this study:

1. We showed evidence that puncture and injection of 40 µl volume into the epineural space does not cause damage to the peripheral nerve. Epineural injection of methylene blue can spread along the nerve, while the dye remains within the epineural compartment.
2. Injection of 1:80 diluted methylene blue solution effectively stains the sciatic nerve in the rat.
3. Injection of 40 µl 1:80 diluted methylene blue solution stains an 18 mm long segment of rat's sciatic nerve.
4. Epineural injection of diluted methylene blue into rat sciatic nerve does not cause functional and structural damage to the stained nerve.
5. Finally, we demonstrated that human cadaveric digital nerves can be stained with epinerual injection of diluted methylene blue solution.

7. Publications related to the thesis:

Szabó T, Kormos V, Rékási Z, Gaszner B. Epineural Methylene Blue Injection May Aid Localization of Digital Nerves in Dupuytren's Surgery. Eur Surg Res. 2021 Oct 22:229-237. doi: 10.1159/000519666. PMID: 34689139. (IF: 1,114)

Szabó T, Kormos V, Gaszner B, Rékási Z. Perifériás ideg epineurális metilénkék festése kadáver kézen. Orv Hetil. 2022; 163(46): 1843-1848. (IF: 0,707, 2021)

8. Poster related to the thesis:

Tamás Szabó, Viktória Kormos, Zoltán Rékási, Balázs Gaszner. Epineural methylene blue injection may aid localization of digital nerves in Dupuytren's surgery (e-poster). IFSSH, IFSHT & FESSH (International Federation of Societies for Surgery of the Hand, International Federation of Societies for Hand Therapy, Federation of European Societies for Surgery of the Hand) Combined Congress London, June 6-10 2022. London

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