Epineural Methylene Blue Injection May Aid Localization of Digital Nerves in Dupuytren’s Surgery

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Abstract

Background: In Dupuytren’s surgery, limited fasciectomy is still the gold-standard treatment. A relatively high risk of iatrogenic nerve injury has been observed especially when the spiral cords of the Dupuytren’s tissue pull digital nerves away from their normal anatomical location. Intraoperative neural marking could facilitate locating the potentially displaced nerves. Hence, surgery could be undertaken more quickly with a lower risk of iatrogenic nerve injury. Objectives: We hypothesize that digital nerves may be stained with methylene blue (MB) in vivo providing a visual aid to distinguish them from Dupuytren’s tissue. We aim to (a) test an in vivo nerve staining technique using MB in a rat sciatic nerve model and to (b) assess the safety of epineural MB injection. Methods: Three experiments were performed: first, the effects of (a) sham surgery, (b) epineural needle insertion, and (c) 40 μL epineural saline injection were tested in the rat sciatic nerve. Second, we determined the (a) histomorphometric localization of the epineurally injected 40 μL 1 m/m% MB stock solution and (b) we tested whether saline dilution (i.e., 1:40, 1:80, and 1:160) of the stock solution does provide optimal blue color upon 40 μL epineural injection. Third, the functional and morphological effect of 40 μL 1:80 diluted MB injection was compared with that of saline, injected into the contralateral sciatic nerve. The functional effects were tested by assessing the pain threshold by using a dynamic plantar esthesiometer (DPA) and by examination of the animal’s gate and paw posture. Sciatic nerves were subjected to histological examination and morphometry to test structural damage. Results: Neither epineural needle insertion nor saline injection caused any functional or morphological changes. Histological examination revealed that the MB stained the epineural compartment. Epineural injection of 40 μL 1:80 diluted MB into the sciatic nerve stained an 18.18-mm segment of the nerve distal to the puncture point. DPA revealed unchanged pain threshold values on the plantar surface of the limbs. Normal gait and foot posture suggested normal motor functions in all groups. No histological changes were seen in the stained nerves, and the nerve fiber density remained unchanged. Conclusion: We demonstrated that in vivo nerve staining with MB is a suitable method to mark nerves without causing detectable negative effect to the stained nerve. Human trials are required to prove the efficacy of the technique in Dupuytren’s disease.

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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 [1–3]. Most frequently, it is an autosomal dominant disease with variable penetrance [1]. Many nongenetic causes may also play a role in the onset and severity of the disease, and the prevalence can approach 30% [4–6]. Recent genome-wide association studies identified causal genes which are responsible for the imbalance of the WNT signaling pathway, abnormal extracellular matrix modification, and inflammation that play a key role in the development of fibromatosis in Dupuytren’s disease [7, 8]. Despite the increase in nonsurgical treatment of Dupuytren’s contracture [9, 10], limited fasciectomy is still the gold-standard treatment. The rate of iatrogenic nerve injury in limited fasciectomy surgery can reach 3.4% [11] affecting the patient’s quality of life. Nerve injury occurs more commonly in case of dislocated digital nerves by the Dupuytren’s spiral cords [12, 13] and in revision surgeries [11, 14]. The challenge during fasciectomy is to identify and protect the digital nerves which can have an altered anatomical position [15, 16] 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 [17–19]. 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 [12]. A simple and safe nerve staining technique, similar to those already applied in parotid [20] and thyroid [21] surgery, could significantly improve the surgeon’s ability to locate and preserve the nerves.

The aim of this study is to identify a simple nerve staining technique which could aid intraoperative location of the digital nerve by improving its visibility and facilitating separation of the nerve from the Dupuytren’s tissue. The rat sciatic nerve was used as a model because its diameter (1–1.5 mm) is similar to that of the human digital nerves [22].

We chose methylene blue (MB) as it is a commonly used stain with a wide range of therapeutic applications [23–26]. MB as a stain has already been successfully used in parotid [20], biliary [27], thyroid surgery [21], and gynecologic oncology [28]; however, in these studies, the dye was either administered intraductally [20, 27], or the operation area was rinsed with the solution [21, 28]. To the best of our knowledge, the efficacy and safety of the direct epineural MB injection has never been tested. Therefore, 3 animal experiments have been designed to evaluate the possible structural or functional damage of the rat sciatic nerve injected with MB.

Materials and Methods

Animals

Twelve-week-old male Wistar rats (Animalab Kft., Vác, distributor of Charles River in Hungary) were housed in a temperature- and humidity-controlled 12-h light-dark cycle environment (lights on at 6 a.m.) in standard polycarbonate cages (400 mm × 250 mm × 200 mm) with 2 rats per cage provided ad libitum with standard rodent chow (CRLT/N, Szindbád Kft., Gödöllő, Hungary, 11 kJ/g) and water. In vivo experiments were permitted by the National Food Chain Safety Office in Hungary (No. BA02/2000-42/2018) upon approval of the Animal Welfare Committee at Pécs University and the National Scientific Ethics Committee on Animal Experimentation in Hungary.

Experimental Design

In order to test the safety and efficacy of epineural injections, 3 experimental steps were designed using the rat sciatic nerve as a model because its diameter (1–1.5 mm) is similar to that of the human digital nerves [22].

Experiment 1

The safety of the injection was tested on 3 groups of rats (n = 5, respectively): (1) the sciatic nerves were visualized and prepared (sham surgery), (2) the nerves were visualized, and 29G injection cannula insertion was performed, and (3) after preparation and needle insertion, 40 µL of saline solution was injected. This volume was found optimal in pilot studies to avoid leakage of the solution and swelling of injected segments.

The primary goal was here to test if these interventions may cause functional damage to the nerve; therefore, the mechanical pain threshold of rats was examined by using a dynamic plantar esthesiometer (DPA) (see below). The secondary endpoint was to determine if any histologically visible changes take place as a result of the sham surgery, needle insertion, or volume injection. Therefore, histomorphological tools were also applied.

Experiment 2

To determine the optimal minimal concentration of MB that provides well-recognizable blue color, besides the stock solution (1 m/m% methylthionin dissolved in sterile pyrogen-free 0.9 m/m% NaCl from the Pharmacy of Clinical Center, Pécs University), saline dilutions (1:40, 1:80, and 1:160) were tested. Forty microliter volume of the solutions was injected into 4–6 nerves, in anaesthetized rats, respectively. We observed how intense the blue color was and which dilution was optimal to obtain ideal blue contrast. Nerves injected with the stock solution were harvested right after surgery for native histological examination to determine the histanatomical localization of the dye.

Experiment 3

Both sciatic nerves were prepared in 6 rats. The left nerve was injected with 40 µL saline while the right one was treated with 40 µL saline solution and 40 µL of 1 m/m% MB stock solution.

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μL of 1:80 MB. In line with experiment 1, the primary goal was here to see the functional effect of the treatment by DPA measurement on the 7th and 10th postoperative days. 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.

Surgery
In all experiments, animals were anesthetized with ketamine (78 mg/kg) and xylazine (13 mg/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 the 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 et al. [29], 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 were visible.

Measurement of Mechanonociceptive Threshold
A dynamic plantar aesthesiometer (DPA; Ugo Basile, Varese, Italy) was used to measure mechanical hyperalgesia. After 2 presurgery control measurements, animals were re-tested on the 7th and 10th postoperative days [30, 31]. Rats were placed into an observation chamber on a mesh platform. The hind paw midplantar surface was tested by a straight metal filament lifting with increasing upward force till paw withdrawal. The stop signal was attained when the animal removed the paw or when the cutoff force of 50 g was reached, but latest after 10 s. Mean pain threshold values were calculated from the average of 3 tests.

Euthanasia, Tissue Collection, and Histology
Rats were euthanized with urethane (2.4 g/kg) and perfused with 0.1m phosphate-buffered saline, followed by 4% paraformaldehyde in Millonig buffer (pH = 7.4). Sciatic nerves were removed, postfixed for 24 h, dehydrated, and embedded in paraffin. Routine hematoxylin-eosin (HE) staining was performed on 5-µm serial sections. Fifty-micrometer sections were also cut from MB-injected nerves in experiment 2 which were HE-stained or examined as native preparations.

Microscopy, Imaging, and Morphometry
Sections were digitalized using a Nikon Microphot FXA microscope (Nikon, Tokyo, Japan). Nerve fibers were counted in a ×40 objective lens field, and their number was corrected for the size of the examined endoneural area by using ImageJ software. Nerve fiber per pixel values in 4 sections were averaged representing 1 nerve in the statistics. The assessment was performed on all images by the same expert who was unaware of the identity of images. A second person evaluated the images also, and the results were used if this confirmed the results by the first observer.

Statistics
All data were presented as mean of the group ± the standard error of the mean. Datasets were tested for homogeneity of variance and normal distribution. In 1 case, to obtain the normal distribution, a square root mathematical transformation was necessary. Pain threshold values in experiment 1 were tested at each time point using 1-way ANOVA with the “treatment” as the categorical predictor. Student’s t test for independent samples was used to assess the data in experiment 3. Alpha was set to 5%. The required sample size was determined by power analysis estimating 20% difference between means of the groups (alpha = 0.05; β = 0.8).

Results

Experiment 1
Effects of sham surgery (Fig. 1a), needle insertion (Fig. 1b), and saline injection (Fig. 1c) on sciatic nerve function were compared. DPA measurements (Fig. 1j) 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 the 7th ($F_{2,12} = 0.40, \ p = 0.69$) and 10th ($F_{2,12} = 3.40, \ p = 0.06$) postoperative days. Unaltered nerve morphology was found in all groups (Fig. 1d–i). No muscle palsy or wound healing problems were observed.

Experiment 2
Injection of 40 µL 1% MB resulted in an immediate strong blue coloring of the sciatic nerve (Fig. 2a, b, online suppl. Video 1; for all online suppl. material, see www.karger.com/doi/10.1159/000519666). Fifty-micrometer native preparations were suitable to visualize the presence of MB in the nerve (Fig. 2d, f). In adjacent HE-stained serial sections (Fig. 2e), we determined that MB accumulated in the epineurial compartment (Fig. 2d, f). We did not detect intraneuronal blue discoloration. To select the lowest concentration of MB still providing a proper labeling, a series of dilutions (1:40 to 1:160) was tested, and injection of 1:80 dilution provided well-recognizable epineurial discoloration for a considerable distance.

Experiment 3
Saline (Fig. 3a) and MB-injected (Fig. 3b) nerves were compared in the same rats. Injection of 40 µL 1:80 diluted

In vivo Epineural Methylene Blue Staining
Fig. 1. Summary of control experiments. Intraoperative images of sham-operated (only visualized and prepared) sciatic nerve (a), insertion of the injection needle into the epineural tissue of the sciatic nerve without volume injection (b), and needle insertion with consecutive injection of 40 μL saline (c). d–f Low-magnification images of hematoxylin-eosin stained preparations of the same nerves. The boxed areas are shown as the higher magnification images in g–i below the respective nerves. j The result of the DPA measurements expressed as the mechanical threshold values expressed in grams. Bar, 100 μm in d–f and 25 μm in g–i. DPA, dynamic plantar esthesiometer.
MB immediately stained an 18.18-mm (range 10.00 mm–30.10 mm) segment distal to the injection site (online suppl. Video 2). First \((p = 0.34)\) and second \((p = 0.34)\) control DPA (Fig. 3g) revealed no baseline differences between limbs. No difference in pain threshold was found on the 7th \((p = 0.68)\) and 10th \((p = 0.21)\) postoperative days. The rats’ foot posture and gait remained normal. HE staining (Fig. 3c–f) revealed no histological changes upon MB injection, and the fiber density was not changed (Fig. 3h, \(p = 0.91\)).
Discussion

We hypothesize that epineural MB injection is a safe method for intraoperative neural staining. The premise being that by staining the nerve, it would ease the technical challenge of separating the digital nerves from the pathological Dupuytren’s cord and from the fibrous tissue caused by previous operations during revision surgery.

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 sensory function, as mechanical pain threshold remained at the baseline level. In experiment 3, we investigated the effects of 1:80 diluted MB and saline injected into sciatic nerves. Consistently, mechanical pain threshold values remained above 46 g, that is considered to be normal above 40 g in neuropathy models [31]. Besides sensory functions, it is important to remember that the rat sciatic nerve contains somatomotor fibers. This is different from the human digital nerves, which are purely sensory, except for sympathetic fibers. The absence of muscle palsy-related foot pronation upon MB injection indicates preserved motor function. Based on the results of Seltzer’s [29] model of neuropathy, muscle palsy and pronation caused by an oversized nerve injury would preclude DPA measurements. Unaltered histomorphology was in full agreement with intact nerve function.

The color of MB was observed exclusively in the epineural connective tissue space using native and HE-stained consecutive serial sections. No diffusion of dye was observed inside the endoneural space suggesting pre-axonal damage. Histogram f shows the results of dynamic plantar esthesiometry (DPA) in grams. The axon density measurement was illustrated in historgam h. Bar, 100 μm in c, d and 25 μm in e, f.

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Statement of Ethics

In vivo experimental procedures in the rat were permitted by the National Food Chain Safety Office in Hungary (Permission No. BA02/2000-42/2018). The license was given based on the scientific approvals of the Animal Welfare Committee at University of Pécs and the National Scientific Ethics Committee on Animal Experimentation in Hungary.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

T.S. performed the surgeries with the assistance of Z.R., V.K., and B.G. V.K. performed the DPA tests. T.S. wrote the first draft of the manuscript. V.K., Z.R., and B.G. supervised and edited the manuscript. V.K. and B.G. performed the histological work and statistics. T.S., V.K., Z.R., and B.G. designed the experiment and prepared figures and video recordings.

Data Availability Statement

All data are available upon request from the corresponding author.

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