ANESTHESIOLOGY

Extrafasicular and Intraperineural, but No Endoneural, Spread after Deliberate Intraneural Injections in a Cadaveric Study

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ABSTRACT

Background: There is confusion regarding the spread of intraneurally injected local anesthetic agents during regional anesthesia. The aim of this research was to deliberately inject a marker that does not leave the neural compartment into which it is injected, and then to study the longitudinal and circumferential spread and possible pathways of intraneural spread.

Methods: After institutional review board approval, we intraneurally injected 20 and 5 ml of heparinized blood solution under ultrasound guidance into 12 sciatic nerves in the popliteal fossa and 10 median nerves, respectively, of eight fresh, unembalmed cadavers using standard 22-gauge "D" needles, mimicking the blocks in clinical conditions. Ultrasound evidence of nerve swelling confirmed intraneural injection. Samples of the nerves were then examined under light and scanning electron microscopy.

Results: Extrafascicular spread was observed in all the adipocyte-containing neural compartments of the 664 cross-section samples we examined, but intrafascicular spread was seen in only 6 cross-sections of two nerves. None of the epineurium, perineurium, or neural components were disrupted in any of the samples. Spread between the layers of the perineurium was a route of spread that included the perineurium surrounding the fascicles and the perineurium that formed incomplete septa in the fascicles. Similar to the endoneurium proper, subepineural compartments that did not contain any fat cells did not reveal any spread of heparinized blood solution cells. No "perineural" spaces were observed within the endoneurium. We also did not observe any true intrafascicular spread.

Conclusions: After deliberate intraneural injection, longitudinal and circumferential extrafascicular spread occurred in all instances in the neural compartments that contained adipocytes, but not in the relatively solid endoneurium of the fascicles.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The intraneural injection of local anesthetics is an accepted cause of nerve injury related to regional anesthesia
- The intrafascicular *versus* extrafascicular spread of local anesthetics is hypothesized to be necessary for nerve damage to occur

What This Article Tells Us That Is New

- Using the ultrasound-guided injection of heparinized blood into the nerves of cadavers, the extrafascicular spread of injectate was observed
- Intrafascicular spread of injectate was rarely observed, making this an unlikely route of nerve damage after accidental intraneural injection

The introduction of ultrasound and a better understanding of the microanatomy of nerves have challenged the common conception that intraneural injection may be harmful.[1] This is despite previous recommendations by pioneers of regional anesthesia who had promoted deliberate intraneural injection.[2,3] It has generally been accepted that longitudinal spread is, among other causes, a major cause of severe and devastating neurologic complications as substances injected intraneurally may reach the spinal column and beyond,[4,5] and it has been suggested in animal experiments by French *et al.*,[6] Moore,[3] and Selander *et al.*[7] that this spread is *via* the nerve fascicles. This concept has been thoroughly embedded in the culture of regional anesthesia at least since the work of French *et al.*[6] in 1948. An entire industry of needle design and pressure-limiting devices[8] developed around this concept of avoiding intrafascicular injections.

Microscopically, the sciatic nerve consists of two nerves: the tibial and common peroneal nerves, surrounded by a common circumneurium (formerly paraneurium)[9–14] (fig. 1). At a position close to the division of the sciatic nerve at the popliteal level, each nerve is surrounded by its own epineurium, which is a constant finding and easily identifiable with ultrasound.[15–17] The median nerve at the elbow region, on the other hand, is a singular nerve with its own epineurium and circumneurium.[15]

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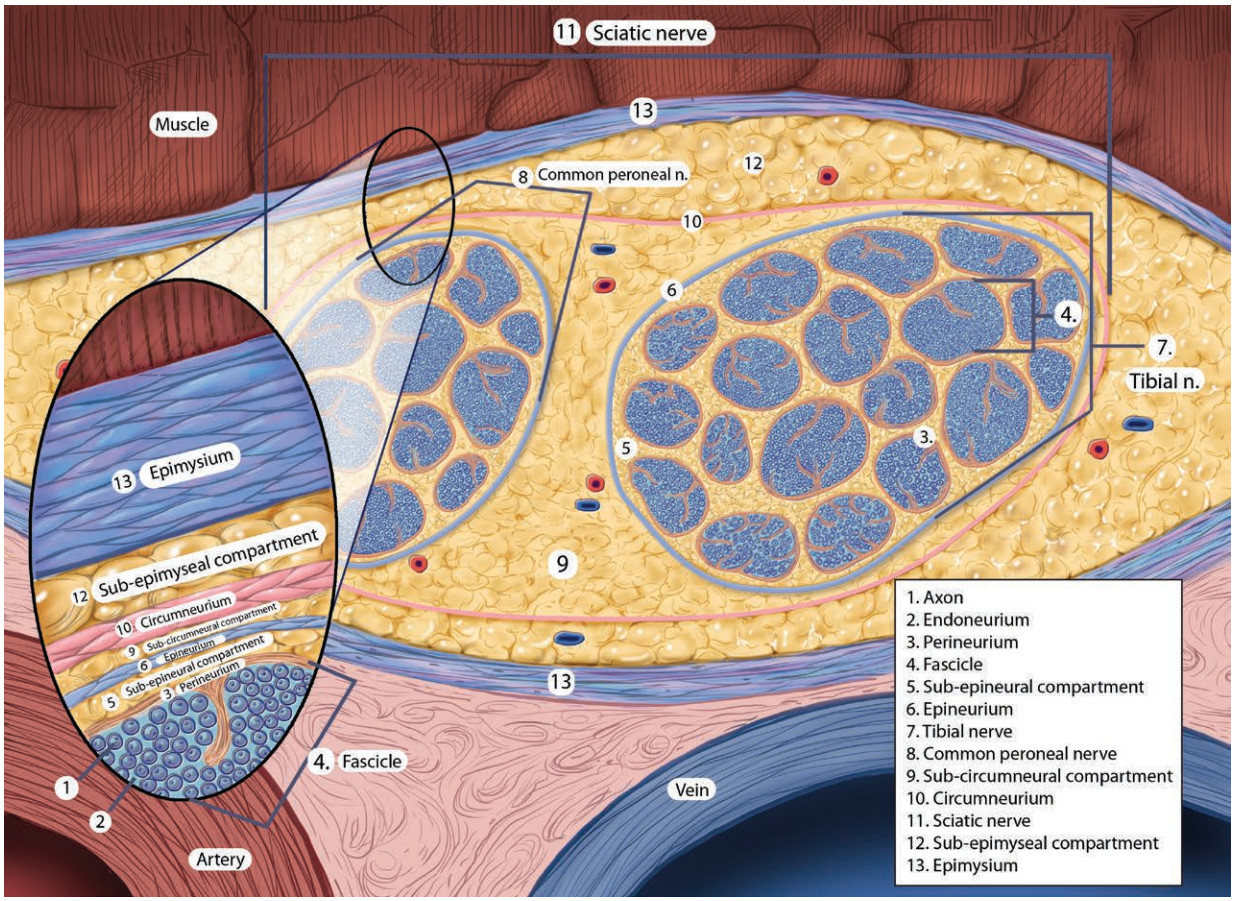

Fig. 1. Schematic drawing of the sciatic nerve at the level of its bifurcation in the popliteal area. The axons (1) are myelinated or unmyelinated and in the endoneurium (2). The axons and endoneurium are surrounded by the perineurium (3) to form a fascicle (4). A bundle of fascicles is, in turn, surrounded by an epineurium (6), which forms a nerve (n.)—the tibial nerve (7) and the common peroneal nerve (8), in this case. Both of these nerves are bundled together by a circumneurium (10; formerly called the paraneurium) to form the sciatic nerve (11) approaching the popliteal fossa. The fascia in which the nerves, arteries, and muscles are housed is the epimysium (13). Each of these layers has a compartment deep in it: the subepimyseal (12), subcircumneural (9), subepineural (5), and subperineural (2) compartments. The last is referred to as intrafascicular, while the subepineurial space is interfascicular (or intraneural). All the compartments except the intrafascicular (subperineural) contain adipose tissue. Reprinted with permission from Mary K. Bryson.

Injection into a singular nerve such as the tibial, common peroneal, or median nerve, *i.e.*, one that is deep to the epineurium, is widely accepted as defining intraneural injection. It is currently generally accepted that an intraneural injection will cause extrafascicular and intrafascicular spread,[18] the latter being associated with major neurologic complications.

Our current nerve block placement technology (mainly non–high-definition ultrasound and nerve stimulation) does not allow us to reliably make the distinction between intrafascicular or extrafascicular injection, nor can it reliably distinguish between subepineurial and subcircumneural injection. Furthermore, the validity of the animal experiment conclusions, widely accepted as factually correct, has recently been questioned on the basis of vast interspecies morphologically differing nerve architecture.[19] If we accept the definition of intraneural injection as deep to the epineurium, the question is whether a fluid injected intraneurally spreads longitudinally and circumferentially and, if it does, in which neural compartments it spreads. Unfortunately, our current understanding has been based on animal studies carried out with markers that do not stay in the compartment into which they are injected.[20–22]

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In an attempt to further explore the disposition of intraneurally injected solutions, we carried out this study with the aim of performing deliberate intraneural injections under ultrasound guidance, mimicking clinical practice, but creating the worst possible conditions for clinical deliberate intraneural injection and then examining the spread in the neural compartments. To achieve this, we used a recently described marker (diluted heparinized blood) that does not leave the compartment into which it has been injected.[23] We then microscopically analyzed the longitudinal and circumferential spread in the extrafascicular and intrafascicular compartments. We also explored other possible intraneural pathways by which this spread could occur.

Materials and Methods

After obtaining Ethics Committee approval from the Grupo Hospital Madrid Clinical Research Ethics Committee (Madrid, Spain; Clinical Research Ethics Committee code 16.03.0922-GHM), we used a recently described solution of diluted heparinized blood[23] as a marker for deliberate intraneural injection in the sciatic nerve at the popliteal level and the median nerve at the level of the elbow in eight fresh, unembalmed, cryopreserved human cadavers. The preparation of this diluted heparinized blood diluted to a hematocrit of approximately 15% has been described elsewhere.[23] In the laboratories of the Departments of Anatomy in the Schools of Medicine from two universities (Centro de Estudios Universitarios [CEU] San Pablo University, Madrid, Spain, and the University of Barcelona, Barcelona, Spain), the deliberate intraneural injection of diluted heparinized blood was performed in 12 sciatic nerves and 10 median nerves of eight cadavers (four per institution). Twenty mL of diluted heparinized blood was injected into the popliteal sciatic nerves under direct ultrasound guidance. The tibial nerve was always targeted, while injections into the subcircumneural space between the tibial and common peroneal nerves were deliberately avoided. For the median nerves, we injected 5 ml of diluted heparinized blood intraneurally—also under ultrasound guidance. Swelling of the nerves was observed and documented by ultrasound to confirm intraneural injection. Injections were done by two practicing anesthesiologists with vast experience in ultrasound-guided regional anesthesia (X.S.-B., E.M.). The needles used were StimuPlex D, 22-gauge (15° bevel; B. Braun Melsungen, Germany). The injections were performed with a syringe drive set at 1,200 ml/h (Alaris, Becton, Dickinson and Company, USA). Injection pressure was measured with a BSmart TM injection pressure monitor (B. Braun Melsungen), and the pressure reached during the injection was kept at or at less than 15 psi to mimic standard clinical practice. In a small number of instances, it reached between 15 and 20 psi, but it never exceeded 20 psi. The pressure values were always higher than those found when the injection occurred outside the nerves.

Thirty to 45 min after the diluted heparinized blood was injected, the samples, including the nerves and all surrounding tissue (fat, muscles, blood vessels, and fascia), were dissected in large blocks. Specimens were immersed in 10% buffered formaldehyde solution for 4 weeks. The blocks were then further sectioned into successive tissue slices of approximately 30-mm × 20-mm slices (4 mm thick), 16 and 14 slices for sciatic and median nerve, respectively, perpendicular to the long axis of the nerves. These tissue slices were processed using paraffin wax and serially sectioned (3 μm in thickness) using a microtome. The sections were stained using hematoxylin and eosin under standard conditions and examined under a light microscope (Leica DM5500 B microscope, Leica SCN Microsystems, Germany) at magnifications ranging from ×8 to ×800. The images were photographically captured (Leica DFC425, Leica SCN Microsystems), and images of the full cross-sections were saved with a scanner (Leica SCN400 Slide Scanner, Meyer Instruments Inc., USA; and Leica SCN Microsystems).

Images of needles were captured with a stereoscopic microscope camera (Leica 56D microscope and Leica EC3 camera, Leica SCN Microsystems) at the same magnification as the nerves and superimposed on a cross-section of tissue images using Adobe Photoshop CC 2017.0.1 version (Adobe Systems Inc., USA).

Samples of a sciatic nerve were dissected from a fifth fresh, unembalmed, cryopreserved human cadaver to evaluate the characteristics of endoneurium tissue within the fascicles and to examine possible endoneurial spaces ("perineural spaces"[3]) under scanning electron microscopy. The different structures were then colored using Adobe Photoshop.

Scanning Electron Microscopy

Slices perpendicular to the long axis of a sciatic nerve section were fixed by immersion for 24 h in 2.5% glutaraldehyde with a phosphate solution buffered at pH 7.28 to 7.32. The slices were then dehydrated through repeated immersions in 50%, 70%, 80%, 90%, 95%, and 100% acetone. The acetone from the samples was exchanged with carbon dioxide in a closed pressurized chamber (Balzers CPD 030-Critical Point Dryer, Bal Tec AG, Lichtenstein) at 31°C until a pressure of 73.8 bars was reached. A carbon layer was then deposited on the samples at a thickness of 0.02 μm with a Balzers MED 010 Mini Deposition System (Bal Tec AG). The evaporating carbon, upon passing an electrical current through a graphite electrode within a vacuum chamber, was regulated to 10^5 millibars. The specimens were covered with a gold microfilm by circulating 20 amperes of electrical current through a gold electrode within a vaporization chamber SCD 004 Balzers Sputter Coater (Bal Tec AG) regulated to a 0.1 millibars vacuum. After this preparation, the specimens were studied with a JEOL JSM 6,400 Scanning Electron Microscope (JEOL Corporation, Japan).

Our primary endpoints included identifying the compartments in which the marker was deposited, the distance of longitudinal spread within extrafascicular and intrafascicular compartments, and the number of fascicles affected by the spread of diluted heparinized blood, as well as searching for other possible pathways of intraneural spread. Secondary endpoints were finding evidence of disruption or morphologic alteration of any of the neural structures (epineurium, perineurium, adipose cells, axons).

Results

The results were obtained from a total of 664 cross-sections. Extrafascicular spread was seen in all samples (figs. 2–4) except in extrafascicular compartments where adipocytes were absent (fig. 5). Intrafascicular spread was absent in all samples except six cross-sections of two of the nerves where "islands" of diluted heparinized blood were observed in the center of the endoneurium of one fascicle at low magnification (figs. 2 and 3). Extrafascicular subepineural spread was found between the fascicles and outside the perineurium, intermingled among the interfascicular adipocytes (fig. 4). The injected diluted heparinized blood cells displaced adipocytes without rupturing or deforming them, and created pathways between them. The epineurium, perineurium, and axons, likewise, were not disrupted or deformed in any sample.

The epineurium, perineurium, and circumneurium formed mechanical barriers that limited diluted heparinized blood spread outside the particular compartments into which it was injected. In several samples, the diluted heparinized blood was found outside and within the neural compartment. This was probably because the needle opening spanned both compartments, and this was not noticed with ultrasound-guided injection. In several cases, the diluted heparinized blood spread into the subepimyseal compartment no deeper than the epimysium.

Furthermore, a large number of diluted heparinized blood cells were observed filling the entire thickness of perineurium between the perineurium cellular layers (fig. 6). The perineurium layers appeared thickened, but we did not measure them pre- or postinjection.

Diluted heparinized blood cells were not found inside some of the fascicles examined (figs. 2 and 3). They were only observed as an "island" of diluted heparinized blood cells that appeared to be in the center of a single sample of a sciatic nerve (University of Barcelona laboratory; fig. 3) and another of a median nerve (CEU San Pablo University laboratory; fig. 2). In both instances, only one fascicle was involved. These were considered intrafascicular injections when examined under low magnification. However, the same two cross-sections studied at high magnification (×800) allowed us to confirm that the erythrocytes had reached the center of the fascicle *via* the perineurium of incomplete septae (fig. 6). This intrafascicular spread could

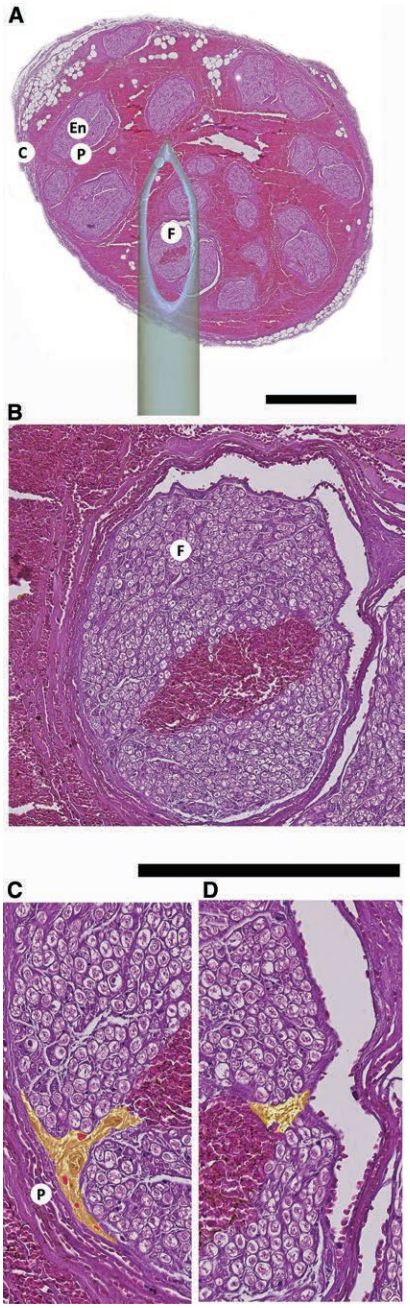

Fig. 2. Microscopic view of an intraneural injection in the median nerve. (*A*) Complete median nerve and the needle used photographed separately and superimposed onto the nerve in postproduction to illustrate its relative size to the largest fascicle (F). Diluted heparinized blood appears to be within the endoneurium (En) of the space inside of the fascicle. (*B*) The same image as *A* at higher magnification showing apparent spread inside the fascicle, as well as spread between the layers of the perineurium (P). (*C* and *D*) Greater magnification that reveals spread between the layers of the perineurium. It also shows spread in the perineurium layer that forms a septum (*yellow*). Magnification: *A*, ×200; *B*, ×400; *C*, ×800; *D*, ×800, Stain: hematoxylin and eosin. C, circumneurium.

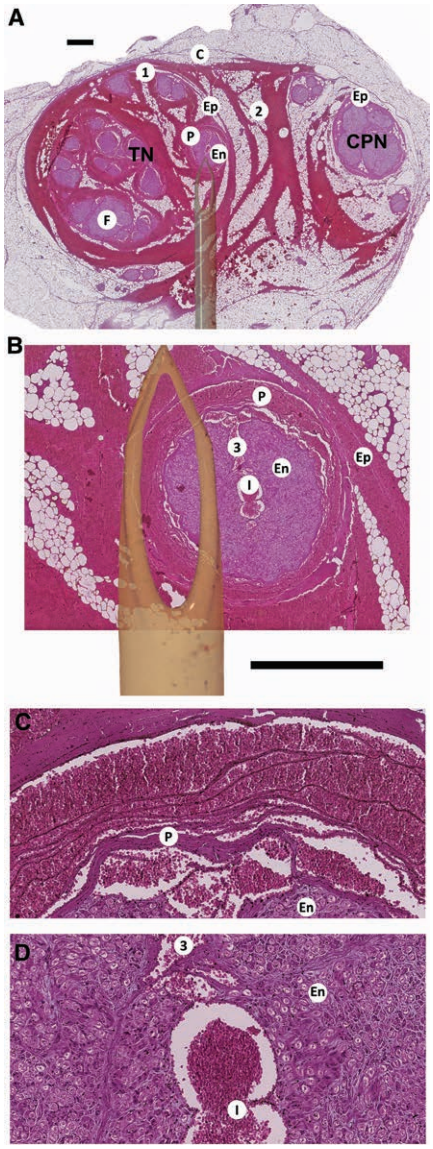

Fig. 3. Microscopic view of sciatic nerve. (*A*) The complete sciatic nerve and the distal end of the needle superimposed on the fascicle (F) in postproduction at the same magnification as the nerve. The diluted heparinized blood (1) can be seen between the fascicles in the compartment deep to the epineurium (Ep) of the tibial nerve (TN) and in the compartments deep to the circumneurium (C; 2). No diluted heparinized blood cells can also be seen in the compartment between the fascicles of the common peroneal nerve (CPN). (*B*) The same image as *A*, at greater magnification. The distal orifice of the superimposed needle is seen to span the fascicles. An "island" (I) of diluted heparinized blood appears to be in the middle of that fascicle and appears to be in the endoneurium (En). (*C*) Diluted heparinized blood is shown spreading through the tissue between the fascicles and between the perineurium layers (P). (*D*) Deposit of diluted heparinized blood inside the fascicle shows "pseudo-intrafascicular" spread at larger magnification. The erythrocytes can be seen inside the innermost perineurial layer of the septum (3). Magnification: *A*, ×40; *B*, ×200; *C*, ×400; *D*, ×400. Stain: hematoxylin and eosin.

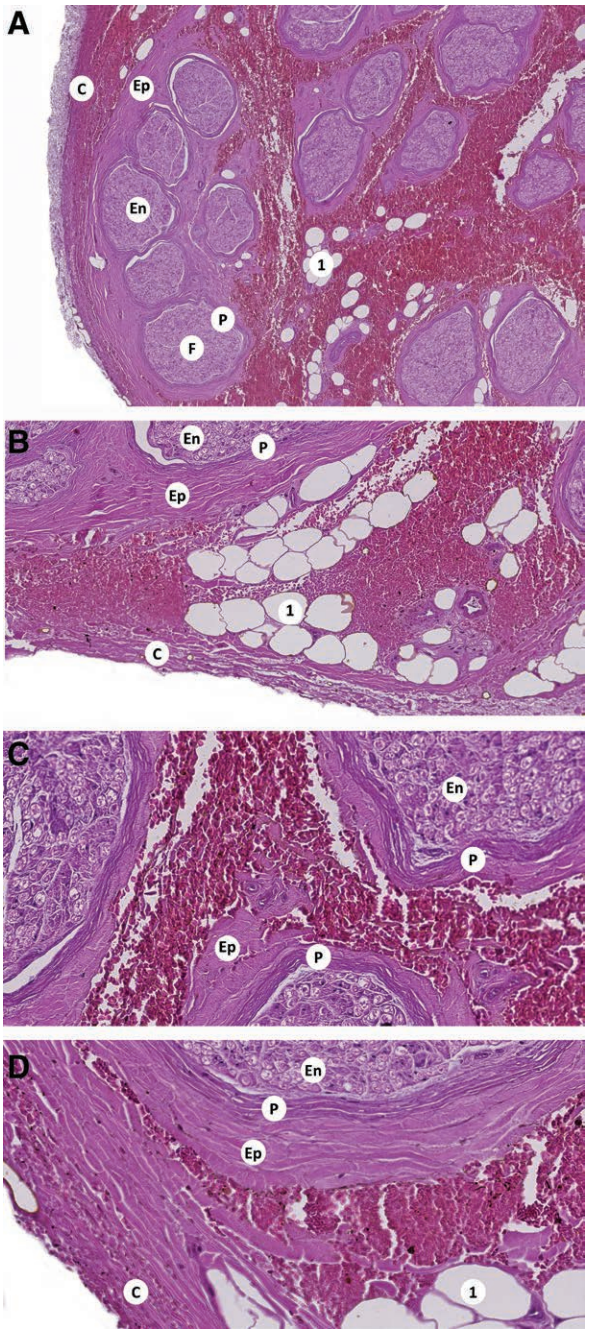

Fig. 4. Microscopic view of intraneural injection into the median nerve at different magnifications. The spread of diluted heparinized blood was limited by the epineurium (Ep). Adipocytes (1) were not ruptured or deformed by the diluted heparinized blood. (*A*) Partial image of the nerve. The marker was localized outside the fascicles and was absent within the fascicles. (*B*) Partial image of the nerve. We can see the marker among the adipocytes, but there are not altered adipocytes. (*C*) Partial image of the nerve. The marker was only found outside the fascicles. (*D*) Partial image of the nerve. The marker was encountered only in contact with adipocytes, collagen fibers that enveloped the fascicles, and the internal surface of the epineurium. Magnification: *A*, ×200; *B*, ×240; *C*, ×500; *D*, ×800. Stain: hematoxylin and eosin. C, circumneurium; En, endoneurium; F, fascicle; P, perineurium.

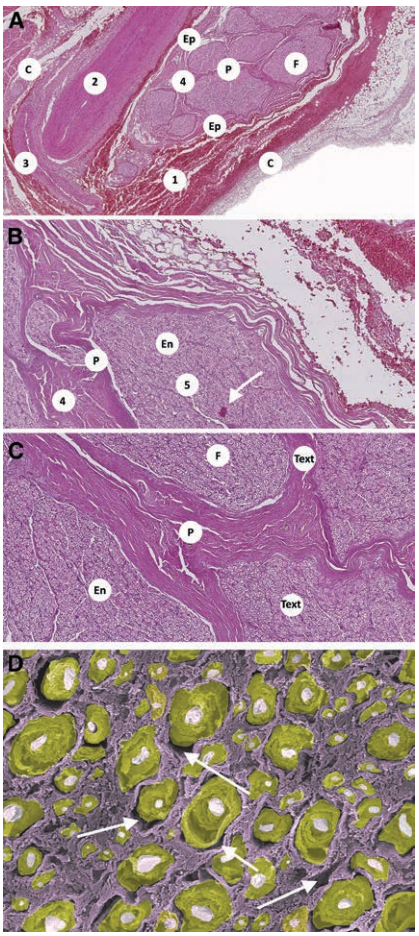

Fig. 5. Microscopic image of deliberate intraneural injection into median nerve. The compact internal structure of the nerve did not permit the injection of diluted heparinized blood between the fascicles (F), into the perineurium, or inside the fascicles. In this case, the entire structure is compact and not only the contents of the fascicles. This occurs due to the absence of adipocytes between the fascicles. This absence prevents the spread of the marker between the fascicles. In this sample, the tissue between the fascicles was formed by collagen fibers without any adipocytes. We can see the complete median nerve with no intraneural diluted heparinized blood. The diluted heparinized blood is completely contained in the subcircumneural space (1), and it did it cross the epineurium (Ep). This is a true subcircumneural injection, which, on ultrasound, appeared similar to intraneural (subepineural) injection. Tissue between the fascicles (interfascicular tissue) is shown as (4). (*A*) The circumneuria (C) include the artery (2), vein (3), and nerve. Diluted heparinized blood spread around all of these structures without penetrating it. This is the neurovascular bundle. (*B* and *C*) Interfascicular (4; between the fascicles) and intrafascicular tissue (5; inside the fascicles—endoneurium [En]) as well as the perineurium (P) did not contain any diluted heparinized blood. Small red spot (*white arrow*) is an artifact—it is translucent. (*D*) Scanning electron microscopy image of the endoneurium of a peripheral nerve. It shows the axons (*white*), myelin sheaths (*light green*), and the endoneurial matrix (*light purple*). What appear like channels or spaces in the endoneurium and between the endoneurium and the myelin sheaths (*white arrows*) are in fact artifacts formed by the processing of the slide. Magnification: *A*, ×40; *B*, ×200; *C*, ×240; *D*, ×1,500. Stain: hematoxylin and eosin.

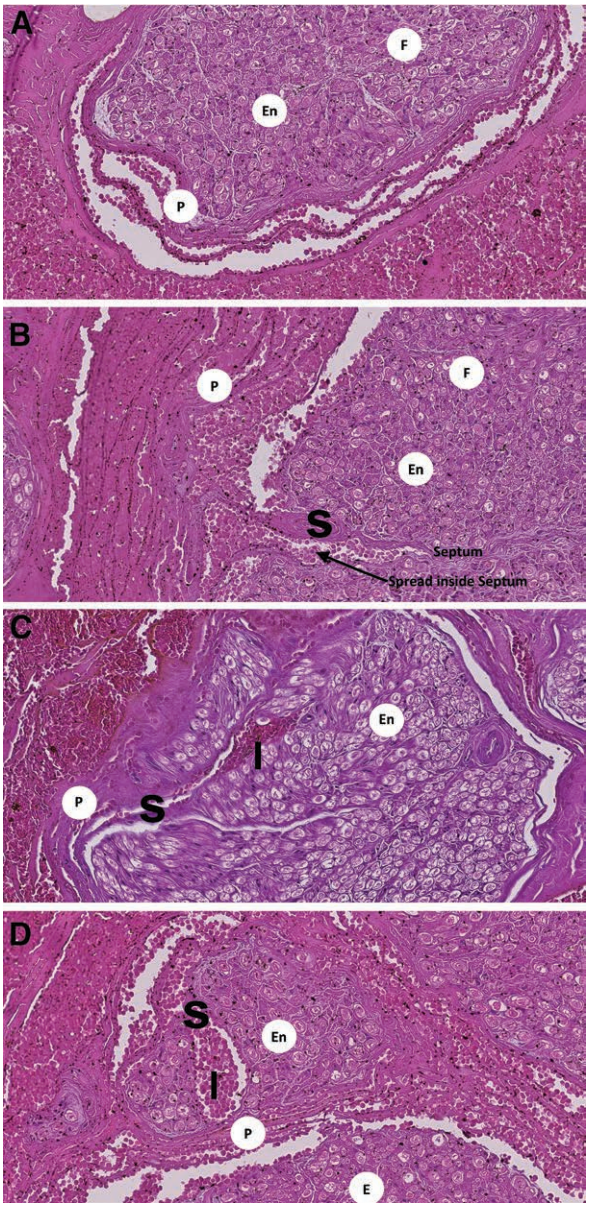

Fig. 6. Microscopic view of a tibial nerve showing diluted heparinized blood spread inside the perineurium (P) layers spread at various magnifications. (*A*) Diluted heparinized blood in tissue between the fascicles that was in contact with the outside of the perineurium. (*B*) The same fascicle (F) as in *A* at higher magnification seen split by a perineurium septum (S). Diluted heparinized blood erythrocytes can be seen among the perineurial layer within the septum. (*C*) Diluted heparinized blood can be seen within the perineurium and perineurium septum filled with diluted heparinized blood. The image of the intrafascicular "island" (I) of erythrocytes represents "pseudo-intrafascicular" (or "pseudo-endoneurial") spread, as it is surrounded by cells of the innermost perineurial layer of the perineurium septum. (*D*) This image shows a fascicle split into several parts by perineurium septa. These septa are filled by diluted heparinized blood. The perineurium septa within the same fascicle have divided the axons into several groups (E). Magnification: *A*, ×200; *B*, ×800; *C*, ×400; *D*, ×800. Stain: hematoxylin and eosin. En, endoneurium.

be identified in three successive cross-sections; in two, the erythrocyte "island" was seen surrounded by an intact perineurium layer—the innermost layer—and in the other one (fig. 6B), it could be identified as a disrupted perineurium layer that allowed spilling erythrocytes to be in direct contact with the axons. The perineuria of these fascicles were fully occupied with diluted heparinized blood cells, including the perineurium of the septae.

After confirming that the perineurium was a possible pathway of spread, we analyzed the perineurium septae in more detail. For the majority of the fascicles visualized in the cross-sections proximal to their separation into two or more fascicles or fascicular interconnections, the perineurium was seen invaginated into the fascicle as an incomplete septum. The lengths of septae increased in successive cross-sections until they formed a complete new fascicle. These septae divided the fascicle into two or more fascicles distally.

In the two samples with apparent intrafascicular spread, the axons were not displaced, injured, or deformed. The erythrocytes did not invade the endoneurium surrounding the axons and did not separate the axons from each other. The erythrocytes were accumulated like an island surrounded by axons (figs. 2 and 3).

The longitudinal spread of the diluted heparinized blood cells that appeared to be inside the endoneurium in these two instances was limited to less than 4 mm in both directions. On the other hand, the longitudinal spread through the adipose tissue outside the fascicles was at least 36 and 28 mm in proximal and distal directions in the sciatic and median nerves, respectively. This distances could have been farther, but they reached the limits of our paraffin wax blocks.

Extrafascicular spread in the sciatic nerves was more longitudinal than lateral or circumferential. The 20 ml of injected diluted heparinized blood did not completely fill the sciatic nerve, and it was observed in clusters closer to the adipocytes located just under the epineurium or adjacent to perineurium. In contrast, the 5 ml of diluted heparinized blood injected into the median nerve completely filled the extrafascicular (subepineural) compartment. The axons did not appear to be altered upon examination of any of the samples.

When we superimposed the needle images taken at a similar magnification as the nerves on the cross-section of nerve images, we observed that the involved fascicles were always smaller than the opening at the distal end of the needle. In previous studies,[9,12] after using specific stains, we also confirmed that hematoxylin and eosin was sufficient to identify and distinguish the endoneurium, perineurium, epineurium, and circumneurium of each nerve.

Under scanning electron microscopy, we found the endoneurium tissue formed by axons of different sizes, a compact structure of intermerged collagen fibers that surrounded each axon individually, and a myelin layer of Schwann cells occupying all space between the axons and endoneurial covers of collagen fibers. We failed to observe any spaces inside the endoneurium (fig. 5).

The results obtained from both laboratories were identical.

Discussion

Intraneural injection has generally been discouraged during regional anesthesia—particularly intrafascicular injections, which have been implicated as the cause of devastating neurologic complications. However, what has not yet been considered is the inability (or at least great difficulty) of injecting a solution into a fascicle, even after deliberately attempting to do so. In fact, it has always been considered a very likely scenario in cases of inadvertent injection into a nerve.[1] Although we do not suggest that the small number of nerves we injected into could be a representative sample, in this research, we attempted to mimic the worst conditions by ensuring direct intraneural injection with large volumes of diluted heparinized blood. On the strength of previous work,[3,6,7] we expected to find a number of fascicles containing diluted heparinized blood and the diluted heparinized blood spreading freely in both longitudinal directions *via* the fascicles (fig. 7A). We also expected that the perineuria and epineuria would be fractured by the created intrafascicular pressure of the large volumes injected. Vlassakov *et al.*[18] recently summarized this hypothetical and severe neurologic complication that anesthesiologists conceptualize: the rupture of the perineurium of the fascicles and extravasation of the injected solution into the extrafascicular compartment after an injection directly into the fascicle. However, we were surprised to observe no evidence of this. The vast majority of fascicles were undisrupted despite the adverse conditions we created for deliberate intraneural injection (fig. 7B). The concepts and proposed pathways of spread that have been defended during the previous decades could not be supported by this study, and a new avenue for future investigation has been created by observing the spread between the cell layers of the perineurium rather than in the endoneurium (fig. 7B).

Another surprise finding was perinerium septa that served as a pathway for longitudinal spread (fig. 7B). This pathway could extend into the endoneurium *via* the perineurium septa also formed by the same perineural cell layers. These pathways have not been described previously to the best of our knowledge.

The striking difference between what we found and what several other researchers found[20–22] may be explained by the fact that the animals they used for their studies (mainly dogs)[21] have vastly different microanatomic nerve architecture than humans,[19] and the marker used in our study has allowed us to visualize microscopic details not possible with the use of india ink or other previous markers.

Conventional ideas and research conclusions were based on four assumptions: (1) that it is possible to inject into the endoneurium of human nerve fascicles because it has been shown to be possible in animal models; (2) that injections

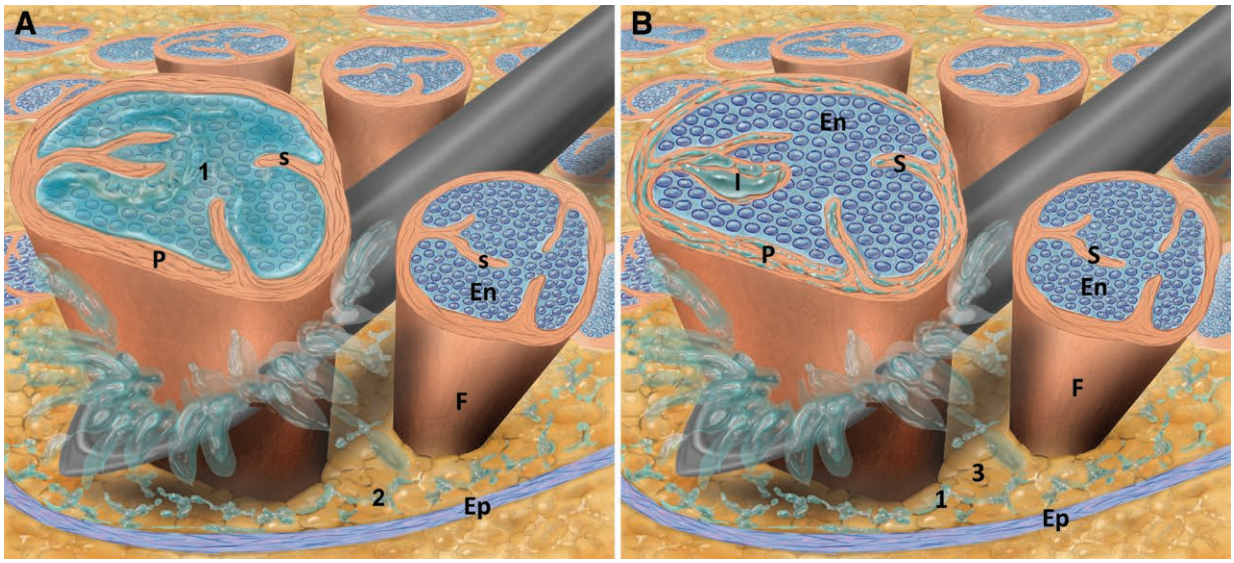

Fig. 7. This is an illustration of intraneural spread. (*A*) The spread that we expected according to the depiction of Vlassakov *et al.*[18] and our previous understanding of the spread inside perineural compartments or fascicles would be intrafascicular (1), extrafascicular in the subepineural space (2), and perhaps extraneural due to back leaking through the needle puncture site. (*B*) What we in fact found, instead, was no spread inside the endoneurium of the fascicles and easy longitudinal and circumferential spread between the collagen fibers of the perineurium, between the fascicles in the space deep to the epineurium, (1) and among the adipocytes (3), and unimpeded spread in the subcircumneural compartment (not illustrated). Spread between the layers of the perineural cells that formed septa appeared like "pseudo-intraendoneural" spread at low magnification. The distal opening of the needle was always larger than the fascicles. Island in perineural cells that form the septum on low magnification appears intraendoneural or inside the endoneurium of the fascicle. Reprinted with permission from Mary K. Bryson.

under or deep into the epineurium—generally accepted as intraneural injections—or intrafascicular injections may cause disruption of neural components; (3) that subcircumneural injection, such as between the two terminal branches of the popliteal sciatic nerve, constitutes an intraneural injection and should be avoided because it can cause nerve injury[24]; and (4) that, as Moore *et al.*[25] stated in reference to the channels inside the endoneurium connnecting the fascicles directly to the central nervous system, "perineural spaces are real and not only imaginary." None of these assumptions could be supported or substantiated by this study. The "channels" that Moore *et al.*[25] observed and referred to as "perineural spaces" are in fact artifacts and not true channels.[26]

The compact endoneural structure and the absence of endoneural spaces that Moore described as "real and not only imaginary"[3] confirmed our observation in this limited number of nerves regarding the impossibility of injecting a fluid directly into the endoneural tissue—the inability of intrafascicular spread. The concept that the axons are delicate, complex structures, imagined as soft, fragile, and easily flooded similar to the collagen fibers of the endoneurium, could not be supported with our study. In fact, in our study, the axons and the endoneurium appeared to form what seemed like a tight and resistant structure that prevents it from being penetrated by injected solutions.

We also observed that the pressure that we applied next to the entry site of the needle was not sufficient to allow longitudinal spread within the fascicle. It was, however, sufficient to cause some backward leakage and longitudinal and circumferential spread outside the fascicles (subepineural space) and in the subcircumneural spaces, as well as between the layers of the perineurium among all layers of perineurium but without exceeding the innermost layer of perineurium, being adipocyte-containing compartments, the only compartments where the spreading of the marker was evident. The spread of diluted heparinized blood in the adipose tissue within the interfascicular compartment and perineurium was met with little resistance, but the extrafascicular spread occurred only if there were adipocytes in the compartments. Perineurium spread through invaginating perineurium septae of the fascicles should thus be viewed as "pseudo-intrafascicular" spread and not as direct intrafascicular (intraendoneural) injections. The disruption of that delicate innermost perineurium layer could, however (at least theoretically), result in true intrafascicular spread.

After reviewing all the cross-sections and the size of the distal needle orifice, it is clear that the fascicles of the nerves that we examined were smaller than the needle orifice, which allowed the injected solution to spread between the perineural layers and the extrafascicular tissue. Further

research using a similar methodology with needles that are of similar or smaller size of the fascicles is required.

In conclusion, if defined as an injection deep to the nerve epineurium, we found that intraneural injection is easily achievable. However, in this study, classically described intrafascicular injection was not observed after deliberate intraneural injection. In the limited number of nerves we examined, it appeared to be impossible to inject diluted heparinized blood cells into the endoneurium. The authors, however, in no way propose or suggest that intraneural injection may be safe because of these findings. Extensive targeted clinical research would be required for such a claim.

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Competing Interests

The authors declare no competing interests.

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