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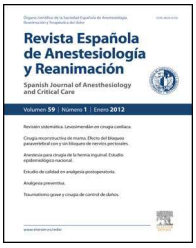

ORIGINAL ARTICLE

Electron microscopy of human peripheral nerves of clinical relevance to the practice of nerve blocks. A structural and ultrastructural review based on original experimental and laboratory data☆

M.A. Reina[a,b,*], R. Arriazu[c], C.B. Collier[d], X. Sala-Blanch[e,f], L. Izquierdo[g,h], J. de Andrés[i,j]

[a] *Department of Clinical Medical Sciences and Applied Molecular Medicine Institute, CEU San Pablo University School of Medicine, Madrid, Spain*
[b] *Department of Anesthesiology, Madrid-Montepríncipe University Hospital, Madrid, Spain*
[c] *Department of Basic Medical Sciences and Laboratory of Histology and Imaging, Institute of Applied Molecular Medicine Institute, CEU San Pablo University School of Medicine, Madrid, Spain*
[d] *Department of Obstetric Anaesthesia, Prince of Wales Private Hospital, Sydney, New South Wales, Australia*
[e] *Department of Anesthesiology and Critical Care, Clinic Hospital, Barcelona, Spain*
[f] *Laboratory of Surgical NeuroAnatomy, Human Anatomy and Embryology Unit, Faculty of Medicine, Universitat de Barcelona, Barcelona, Spain*
[g] *Department of Clinical Medical Sciences, CEU San Pablo University School of Medicine, Madrid, Spain*
[h] *Department of Vascular Surgery, Madrid-Montepríncipe University Hospital and Madrid-Torrelodones University Hospital, Madrid, Spain*
[i] *Anesthesia Department, Valencia University Medical School, Valencia, Spain*
[j] *Multidisciplinary Pain Management Center, Department of Anesthesiology and Critical Care, Valencia University General Hospital, Valencia, Spain*

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KEYWORDS
Nerve;
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Scanning electron microscopy;

Abstract
Aim: The goal is to describe the ultrastructure of normal human peripheral nerves, and to highlight key aspects that are relevant to the practice of peripheral nerve block anaesthesia.
Method: Using samples of sciatic nerve obtained from patients, and dural sac, nerve root cuff and brachial plexus dissected from fresh human cadavers, an analysis of the structure of peripheral nerve axons and distribution of fascicles and topographic composition of the layers that cover the nerve is presented. Myelinated and unmyelinated axons, fascicles, epineurium, perineurium and endoneurium obtained from patients and fresh cadavers were studied by light microscopy using immunohistochemical techniques, and transmission and scanning electron

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* Corresponding author.
E-mail address: miguelangel@perticone.e.telefonica.net (M.A. Reina).

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Transmission electron microscopy;
Immunohistochemistry

microscopy. Structure of perineurium and intrafascicular capillaries, and its implications in blood–nerve barrier were revised.
Results: Each of the anatomical elements is analyzed individually with regard to its relevance to clinical practice to regional anaesthesia.
Conclusions: Routine practice of regional anaesthetic techniques and ultrasound identification of nerve structures has led to conceptions, which repercussions may be relevant in future applications of these techniques. In this regard, the ultrastructural and histological perspective accomplished through findings of this study aims at enlightening arising questions within the field of regional anaesthesia.

PALABRAS CLAVE
Nervios;
Fascículos;
Endoneuro;
Perineuro;
Epineuro;
Microscopia electrónica de barrido;
Microscopia electrónica de transmisión;
Inmunohistoquímica

Microscopía electrónica de nervios periféricos humanos con relevancia clínica en los bloqueos anestésicos periféricos. Una revisión experimental de la estructura y ultraestructura basada en nuevos resultados del laboratorio

Resumen
Objetivo: El principal objetivo fue describir la ultraestructura normal de los nervios periféricos y aportar todos aquellos aspectos relevantes para la práctica de los bloqueos anestésicos de los nervios periféricos.
Método: A partir de muestras de nervio ciático obtenidas de pacientes y muestras de saco dural, manguitos durales y nervios del plexo braquial disecados de cadáveres frescos se estudió la estructura del nervio periférico, incluyendo los fascículos y su composición topográfica, como así también la morfología de las cubiertas que revisten el nervio. Axones mielinizados, no mielinizados, fascículos, epineuro, perineuro y endoneuro fueron estudiados por microscopía óptica incluyendo métodos de inmunohistoquímica, y por microscopía electrónica de barrido y de transmisión. La estructura del perineuro, de los capilares intrafasciculares y su implicación en la barrera hematonerviosa fueron revisados.
Resultados: Cada uno de los elementos anatómicos fue analizado de forma individual, y los resultados fueron correlacionados con su impacto en la práctica clínica de la anestesia regional.
Conclusiones: La práctica cotidiana de técnicas de anestesia regional y la visualización ecográfica de las estructuras nerviosas nos llevan a crear interpretaciones de la anatomía de los nervios cuyas repercusiones pueden ser importantes en la futura aplicación de la técnica. A este respecto, los resultados obtenidos en estudios ultraestructurales e histológicos están dirigidos a dar respuestas a esas dudas con origen en el entorno de las técnicas de la anestesia regional.

Introduction

Peripheral nerves transmit electrical signals from the periphery towards the central nervous system and provide sensory, motor, secretory or vegetative functions. Most peripheral nerves are mixed, containing axons of motor and sensory neurons; some also contain neurons of the autonomic nervous system.[1] Peripheral nerves are surrounded by epineurium, perineurium and endoneurium. Axons are the essential transmitting units of peripheral nerves, and are surrounded by endoneurium. Multiple axons are grouped into fascicles, which are surrounded by perineurium. Fascicles in turn, can be grouped in fascicular bundles, which are covered by epineurium.

Traditionally, these concepts had been learnt from artistic drawings or diagrams. However, conventional descriptions may be incomplete and may not provide answers to several questions such as whether they have a role of epineurium and endoneurium in establishing an effective barrier for the enclosed axons or to which extent each of these layers contribute to limit the passage of local anaesthetics. Other considerations include whether, for a single nerve, the number of fascicles remains constant or decreases as it reaches distal areas. Are axons invariably placed the same fascicle along its entire length? Answers to these questions may help to rationalize events arising in clinical practice such as whether onset time of motor and sensory blockades related with specific location of axons inside nerves. Neurological damage caused by intraneural injection of local anaesthetics varies[2] depending on where it occurs at endoneural, perineural or epineural level. In relation to the needles used in regional anaesthesia, the shape of lesions caused by different types of needle tips such as short bevelled may lead to more severe neuronal damage if local anaesthetics are injected inside neural fascicles. Endoneurium has been shown to play a role in restoring dural lesions caused by needle tips. Interestingly, there are few studies on the histology of nerves published in anaesthesia journals. Detailed nerve structure transcends the daily clinical practice of regional blockade, which has been based mainly on identification of macroscopic anatomical features such as the existence of neural layers, neural

fascicles and axons, while other important issues such as those aforementioned in relation to regional anaesthesia may require further discerning. A number of anatomical terms continue to cause confusion owing to ambiguities as the same word may refer to different structures. Such is the term ''epineurium,'' as it often constitutes a source of discussion in clinical practice.[3] In some circumstances it may refer to the outer and most peripheral portion of connective tissue surrounding nerves or it may otherwise, refer to both epifascicular and interfascicular portions of connective tissue outside fascicles. The earlier definition of epineurium serves the purposes of this review. Since histological books do not provide answers to several of these questions, we set up an experimental study focusing on issues relevant to anaesthetic practice, examining human nerve tissue samples and looking for evidence in structures which have been extensively described. Similarly, by means of systematic examination of morphological aspects we aimed at anticipating part of the answers in regards to neural blockade and accidental nerve damage.

Knowledge of the anatomical distribution of nerves and their relationship to neighbouring structures is important for successful practice of peripheral nerve block (PNB) techniques.[4–6]

The goal of this study is to describe new findings and to confirm others in regards to the histology of normal human peripheral nerves, and to highlight key aspects that are relevant to the practice of PNB anaesthesia.

Materials and methods

Following approval from the Madrid Hospital Group Clinical Research Ethical Committee, samples of sciatic nerves were obtained after surgical knee amputation (supracondylar amputation of lower limbs), from 18 patients aged 55–85 years. Samples of dural sac, nerve root cuff and brachial plexus were also obtained from four fresh human cadavers. Patient consent for the tissue donations and other procedures included in this research were obtained. Patients suffering from neurological or endocrine (diabetes mellitus) diseases were excluded from the study.

The sciatic nerve was located by inspecting the area posterior to the ligated femoral vessels, and was isolated from the surrounding tissue using blunt dissection. The sciatic nerve and the tibial and common fibular nerves were dissected via a postero-lateral approach until the sciatic nerve itself was exposed. The dissection began from the anterior portion of the nerve, and continued as far cephalad as possible. Tissue samples were collected and prepared for each of the microscopy techniques in a room adjacent to the operating room and were later transferred to the selected specific laboratory. Cross-sections perpendicular to the axis of the nerve were sampled and studied by light microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and by immunohistochemical methods (IMCh). The brachial plexus nerves from four fresh human cadavers were included for microscopic study of peripheral nerves from their origin in the spinal canal. These samples extended from the dural sac and nerve root cuff for a distance of approximately 8 cm.

Preparation for light microscopy

The sciatic nerves were fixed by immersion in 10% formaldehyde solution for 48 h. Samples were then dehydrated at increasing concentrations of ethanol, and paraffin embedded for conventional histological diagnosis. Five-μm-thick sections for immunohistochemistry and routine stain (haematoxylin-eosin, Masson's trichrome, and orcein) were made in each sample ($n = 38$).

Preparation for immunohistochemical methods (IMCh)

Samples of sciatic nerve ($n = 22$) were processed for immunohistochemical methods.

Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% in phosphate-buffered saline (PBS) pH 7.4, to block endogenous peroxidase. Pretreatment of sections by heat in citrate buffer (using a pressure cooker) was performed to enhance the immunostaining. Sections were incubated with a monoclonal mouse anti-EMA (Epithelial membrane antigen) (Dako, USA, ready to use). All primary antisera were diluted in PBS pH 7.4 containing 1% bovine serum albumin (BSA) plus 0.1% sodium azide. All incubations with primary antisera were kept overnight at 4 °C. The second day, immunohistochemistry was performed with standard procedures using Histostain® Bulk Kit (Zymed, South San Francisco, CA, USA).

The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3′,4,4′-Tetraminobiphenyl, Sigma, St. Louis, USA) in 200 ml of PBS, plus 40 μl hydrogen peroxide.

After immunoreactions, sections were counter-stained with Harris haematoxylin, dehydrated in ethanol, and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany). The specificity of the immunohistochemical procedures was checked by incubation of sections with no immune serum instead of the primary antibody.

Digital images of light microscopic fields of perineurium were acquired with a Leica DM6000, Leica Digital Camera DFC425 and analyzed with MetaMorph software (Leica MMAF 1.4).

Preparation for TEM

Samples of sciatic nerve ($n = 36$) were processed for transmission electron microscopy immediately after surgery. The specimens (three samples per patient) were cut at 1 mm-thick × 2 mm-longitudinal segments and fixed for 24 h in a solution of glutaraldehyde 2.5% and a buffer phosphate solution at a pH of 7.2–7.3. They were later fixed with a solution of 1% osmium tetroxide and 1% ferrocianure for 1 h. The specimens were dehydrated with increasing concentrations of acetone in water and soaked in epoxy resin (Epon 812). The resin was polymerized at 60 °C for 24 h. Thin slides, 0.5 μm thick, were dyed with Richardson's methylene blue and observed by light microscopy. Ultra thin slides, 70 nm thick, were cut with an ultra microtome (Reichert Jung ultracut E, Viena, Austria) and treated with Reynold's lead citrate solution over three min. The specimens were

then observed under a Jeol 1010 Transmission Electron Microscope (JEOL Corp. Ltd., Tokyo, Japan). The images were analyzed with measure IT software from Olympus, available free at http://www.olympus-sis.com.

Preparation for SEM

Transverse cross-sectional cuts of sciatic, tibial and peroneal nerves were used, for scanning electron microscopy. In total, 60 samples (5 samples per patient) were prepared. Samples had a thickness of 5 mm and were labelled with sutures to identify their orientation.

Samples were fixed by immersion for 24 h in 2.5% glutaraldehyde with a phosphate solution buffered at a pH of 7.28–7.32, and then dehydrated through repeated immersion in acetone 50, 70, 80, 90, 95% until a concentration of 100% was reached. The acetone from the samples was exchanged with carbon dioxide in a closed pressurized chamber (Balzers CPD 030-Critical Point Dryer, Bal Tec AG, Fürstentum, Lichtenstein) at 31 °C until the critical pressure of 73.8 bar was attained.

Tissue samples were mounted on metal sample holders of 25 mm diameter. A carbon layer of less than 200 Armstrong in width was deposited over the samples with a Balzers MED 010 Mini Deposition System. The carbon layer evaporated on passing an electrical current through a graphite electrode within a vacuum chamber regulated to 10−5 millibars. The specimens were covered with a gold microfilm, by circulating a 20 amp current through a gold electrode within a vapourisation chamber SCD 004 Balzers Sputter Coater regulated to 0.1 millibars vacuum. Afterward, the specimens were studied with a JEOL JSM 6400 Scanning Electron Microscope (JEOL Corp. Ltd., Tokyo, Japan).

Results

Unmyelinated and myelinated axons are present within the same nerve fascicles of mixed nerves and their respective locations may vary along the nerve. In myelinated axons, there is a direct relationship between the diameter of the axon and the thickness of myelin (Fig. 1A). Large axons have thicker myelin because a larger number of coating layers. The myelin sheet results from the superposition of several layers of the plasmatic membrane of the Schwann cells. In unmyelinated axons the Schwann cells (Fig. 1B) take a central position within a group of unmyelinated axons that are arranged at their periphery. Strings of cytoplasm of a single Schwann cell surround and separate individual unmyelinated axons in its vicinity, and surround every axon in an incomplete way.

This allows a single Schwann cell to bind together six to twenty axons (Fig. 1B). These prolongations of cytoplasm also surround packages of collagen fibres that provide greater mechanical strength to the whole system. The diameter of unmyelinated axons varies between 0.2 and 0.8 μm.

It might be expected that axons with shorter pathways would be placed more superficial within nerve fascicles, while more central ones would correspond to axons travelling to distant locations, such as it occurs in spinal cord. However, this is not the case in peripheral nerves. Inside nerve fascicles there is an intraneural plexus, in which the

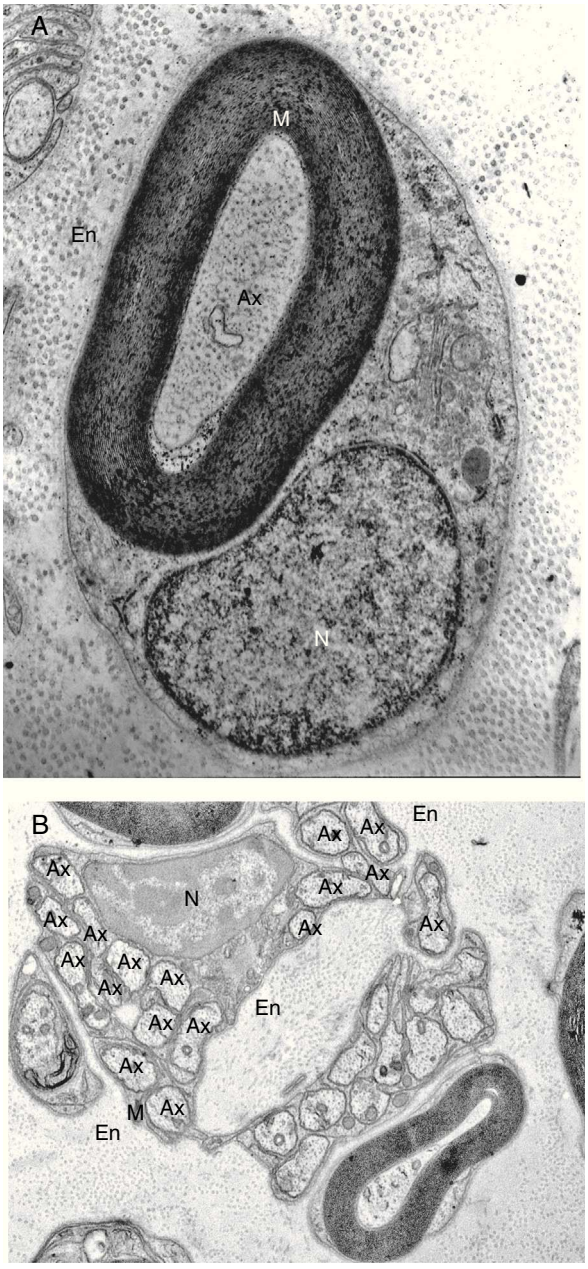

Figure 1 Myelinated and unmyelinated axons. (A) Myelinated axon within a peripheral nerve. Ax = axon, M = myelin sheath, N = nucleus of Schwann cell, En = endoneurium. TEM. Magnification 12,000×. (B) Unmyelinated axons (Ax) within a peripheral nerve. N = nucleus of Schwann cell, M = adjoining myelin sheath, En = endoneurium. TEM Magnification 20,000×.

axons take different positions in different fascicles along its path (Fig. 2A–D). Successive cuts, which have been taken every few millimetres along a nerve show that the topographic map of the fascicles varies.

Variations in topography of fascicles along a nerve are explained by the interchanging of axons between different fascicles (Fig. 2B and C). This transfer of axons begins in the proximal region of the spinal ganglion (Fig. 2D), close to the epidural space, and continues along the intervertebral canal. The portion of nerve roots which extends from the point within their epidural space and inside the dural sleeve

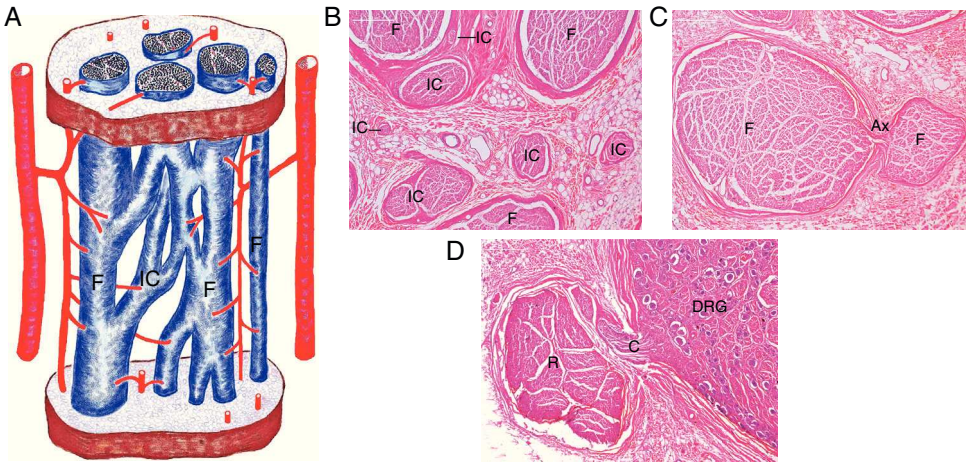

Figure 2 Intraneural plexus. (A) Diagram of an intraneural plexus within a peripheral nerve. F = fascicles, IC = interfascicular connection of axons (as found in branches of the brachial plexus). (B) Intraneural plexus within a peripheral nerve, from the brachial plexus. IC = interfascicular connection of axons. F = fascicles. Haematoxylin-eosin. (C) Intraneural plexus within a peripheral nerve. Interfascicular connection of axons (Ax) between 2 fascicles (F), obtained from the brachial plexus. Haematoxylin-eosin. (D) Sample obtained 1 mm out from the dural sac. M = motor root, S = sensory roots, D = dura, c = interconnecting axons, bv = blood vessel. Haematoxylin-eosin.
(A) With permission of author. Published in Ref. 43.

to the beginning of the spinal ganglion, these contain mainly axons from the anterior and posterior roots that are largely separated from one another, but with a few interconnecting axons. Alongside the ganglion, the passage of groups of axons increases towards the anterior root, with both roots immersed within the dural sleeve inside the foramen. At the distal end of the ganglion, the fascicular structure seen in peripheral nerves starts to appear. The number of fascicles increases along the course of the nerves. In mixed nerves, single fascicles, groups of fascicles and 'small' fascicles may be found (Fig. 2B and C). The latter form a characteristic structure known as ''fascicular interconnections''. Inside peripheral nerves, fascicular interconnections exhibit varying numbers of axons, which diameters are consistently thinner in comparison with axons from connecting peripheral nerves. In few samples, axons from fascicular interconnections can be seen traversing the thickness of the perineurium of their respective originating fascicles (Fig. 3A–C).

Endoneurium enclosing myelinated and unmyelinated axons consists mainly of collagen fibres (Fig. 1A and B). Collagen in the endoneurium is similar to collagen found in the perineurium, and is probably produced by Schwann cells, where a high proportion of collagen is found. TEM allowed the examination of collagen fibres present at the conjunction of axons and Schwann cells. Conversely, SEM enabled accurate observations of the structural array of these collagen fibres, which shape genuine tunnels for each axon (Fig. 4A). In the samples examined, all tunnels contained a single myelinated axon, whereas unmyelinated axons and capillaries lacked from these collagen formations. TEM permit us can see unmyelinated and endoneural capillaries (non-fenestrated capillaries) (Fig. 4B).

Perineurium covering each fascicle forms a continuum around each fascicle, and is composed of concentric cellular layers and collagen fibres interposed among these layers. In sciatic nerves, perineurium is composed of 8–18 alternating concentric layers of cells and connective tissue (Fig. 5A–E).

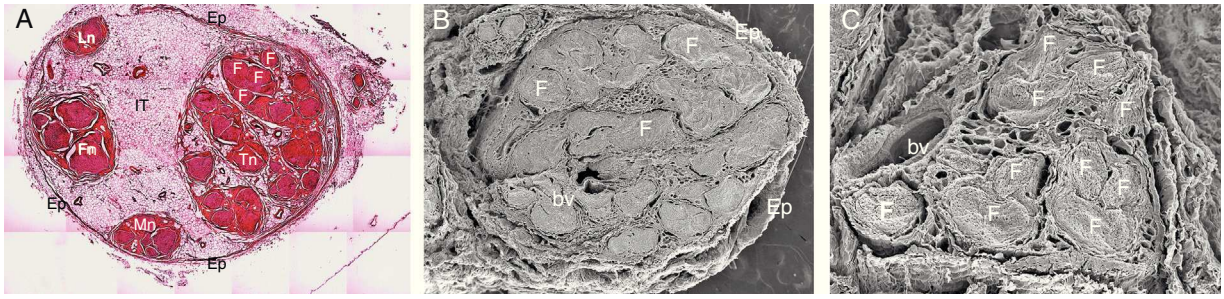

Figure 3 Sciatic nerve. (A) Sciatic nerve sample at the popliteal region. Tn = tibial nerve component, Fn = common fibular nerve components, Mn = medial sural cutaneous nerve, Ln = lateral sural cutaneous nerve. F = fascicles. Ep = epineurium. Haematoxylin-eosin. (B) Sciatic nerve sample at the popliteal region. F = fascicles, bv = interfascicular blood vessel, Ep = epineurium SEM. Magnification 25×. (C) Fascicles surrounded perineural layers, F = fascicles, bv = interfascicular blood vessel. SEM. Magnification: (A) 85×.

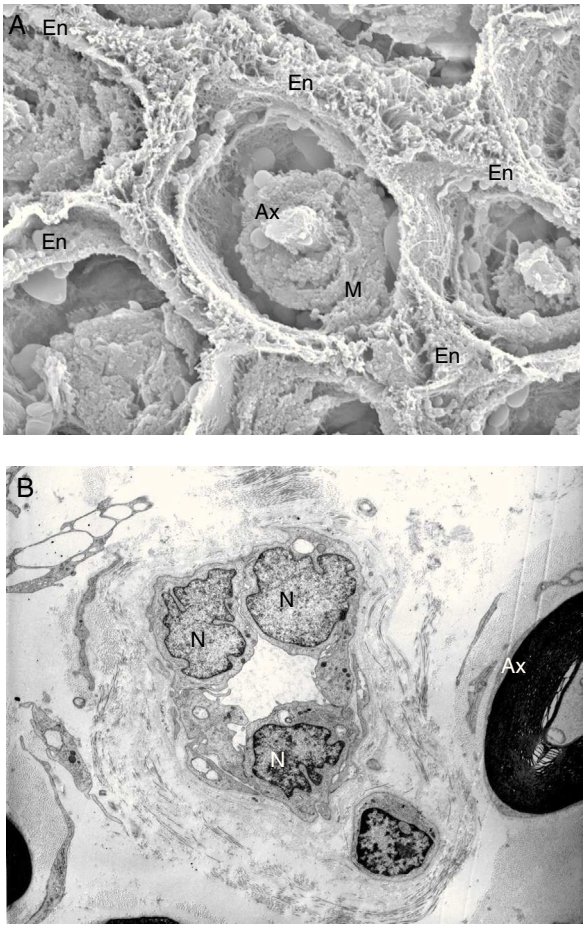

Figure 4 Endoneurium and capillaries inside fascicles of peripheral nerve. (A) Endoneurium M = myelin, Ax = axon, En = endoneurium SEM. Magnification 3300×. (B) Non fenestrated capillary inside fascicle closed to myelinated axons. N = nucleus of endothelial cell. TEM. Magnification: 3000×.

Overall, the number of perineural cell layers bears a linear relationship to the diameter of the fascicles, being higher in fascicles of larger diameter and lower in the fascicles of smaller diameter. We observed numerous intracytoplasmic pinocytotic vesicles in perineural cells, mainly in the outer layers (Fig. 5A).

The thickness of the perineurium is typically 7–20 μm and each layer of the perineurium is formed by single cells joined together. Perineural cells have a thick basement membrane, which surrounds the perineural cell layers on both sides, tight junctions and desmosomas, which connect adjacent cells within each layer of perineurium (Fig. 5E).

Perineural spaces among layers of perineurium are composed of fundamental amorphous substance, collagen fibres with few fibroblasts. These collagen fibres can be aligned in different directions, but predominantly in a longitudinal fashion along the axis of the fascicle (Fig. 5E). Few samples showed a collagen structure known as fibrous long spacing (FLS) in perineurium, specifically among collagen fibres placed between perineural layers (Fig. 5B). This structure has different appearance in comparison with common collagen. FLS has a major banding periodicity ranging between 100 and 150 nm and minor bands are absent or sparse as a rule. It contains longitudinally oriented filaments, which are clearly seen traversing the length of the fibril. These fibres have diameters ranging from 675 to 765 nm (Fig. 5B).

Immunohistochemical studies with EMA (epithelial membrane antigen) have been used to differentiate the nature of the tissues surrounding the fascicles. EMA immunoexpression is positive with perineural cell surfaces and negative with collagen fibres. It has been found that single fascicles are surrounded only by perineurium, whereas groups of two or more fascicles are covered by both collagen fibres and some isolated fibroblasts, a structure similar to the epineurium.

Using Masson's Trichrome dye technique, a large number of collagen fibres were identified in epineurium as well as inside layer enclosing groups of fascicles. On the other hand, orcein dye technique enable identification of few elastic fibres in the samples examined.

We have been able to identify 5–12 layers of perineurium using SEM depending on the size of the fascicle (Fig. 5C and D). The perineurium constitutes the axons unique insulating cover and separates them from other nerve components. The outer surface of each perineural layer is formed by the cytoplasmic membranes of perineural cells has small undulations and its appearance is smooth and bright (Fig. 5C and D)

The epineurium (also known as epi-epineurium) is composed mainly of collagen fibres and a few blood vessels with virtually no fat (Figs. 3A, B and 6A, B). Collagen fibres of the epineurium are similar in size and appearance to collagen fibres in dura mater or dural nerve roots, and thickness of the epineurium varies substantially between nerves in various locations.

The epineurium consists of thicker, condensed layers of connective tissue, surrounding nerves externally and tethering them to neighbouring structures, typically muscles and connective tissue. A structure known as ''interfascicular tissue'' (also known as interfascicular epineurium), appears wrapping around fascicles and its thickness increases around groups of nerve fascicles (Fig. 3A). Interfascular tissue is mainly composed of adipocytes, blood vessels of various sizes such as arteries, veins, arterioles, venules and fenestrated capillaries; as well as lymphatic vessels and small nerve endings supplying the vessels (Fig. 3A and B). Interfascicular tissue has elastic properties. The amount in each nerve fascicle varies, although it is proportionally higher in larger nerves.

The ''intrafascicular tissue,'' is a structure tightly enveloped by perineurium and composed by nerve axons, Schwann cells, endoneurium and non fenestrated capillaries (Figs. 3A,B, 4A, B and 5C).

Fenestrated capillaries reach the outer layers of the perineurium enclosing fascicles; thereafter capillaries becomes non-fenestrated.

It is known that non-fenestrated' type of capillaries contribute to the barrier effect of membranes (Fig. 4B). The precise location where fenestrated extra-fascicular capillaries become non-fenestrated intrafascicular capillaries has not been previously described. This has been identified in our samples.

Discussion

Results obtained from human samples allow clarification of previous concepts in regards to peripheral nerves at

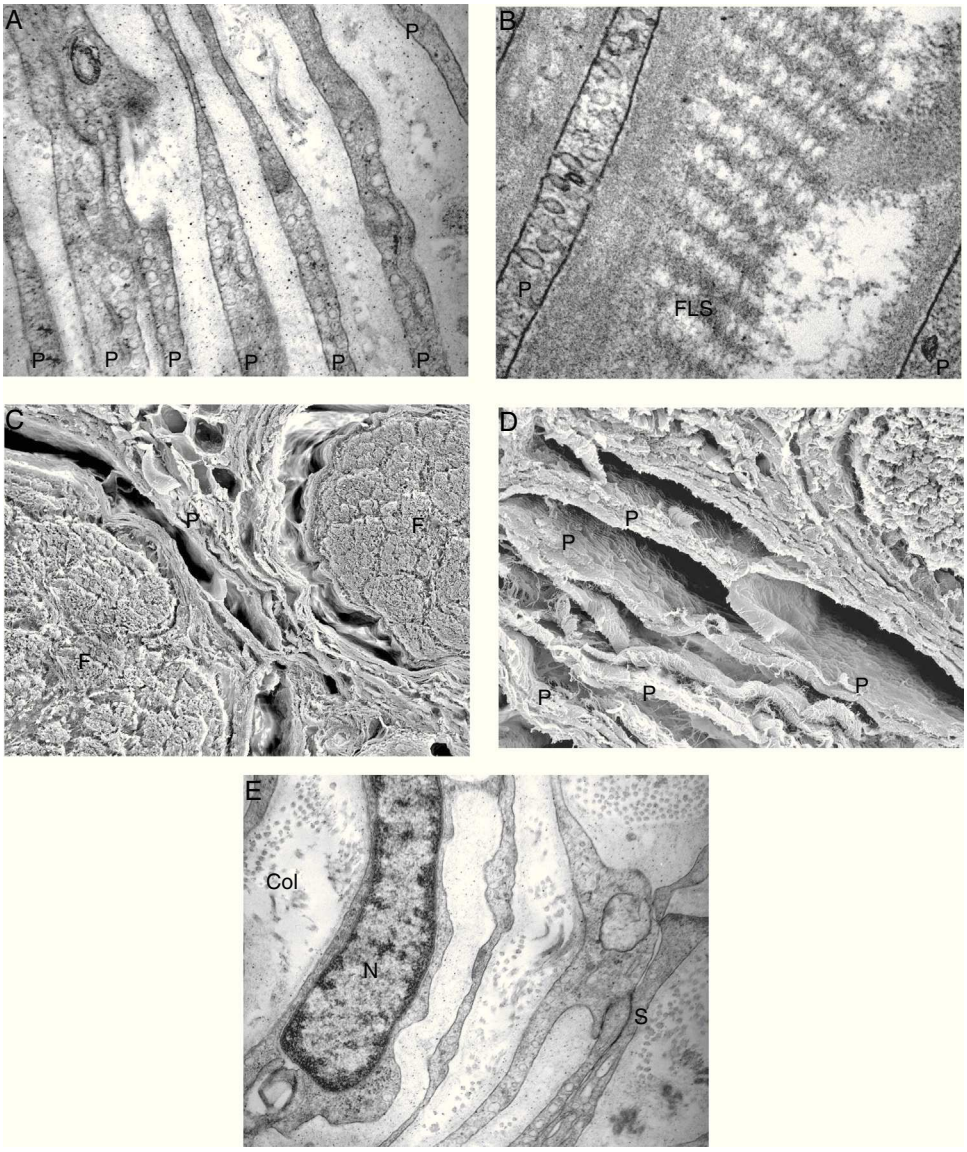

Figure 5 Perineurium in a peripheral nerve. (A) P = perineurial layers and specializated junctions. TEM. Magnification: 30,000×. (B) Fibrous long spacing (FLS) inside perineurial layers, P = perineurial layers. TEM. Magnification: 100,000×. (C) Tridimentional detail of perineural layers F = Fascicles surrounded by perineural layers (P). SEM. Magnification 150×. (D) Tridimentional detail of perineural layers. P = perineurial layers. F = Fascicle. SEM. Magnification 500×. (E) Perineurial layers and specializated junctions. N = Nucleus of perineural cell, Col = Collagen fibres, S = Specialized junctions.TEM. Magnification: 20,000×.

ultrastructural level, which in turn contribute to better understand neural lesions inflicted by accidental intraneural injection or as consequence of inadvertent nerve damage caused by needle tips.

Historically, regional anesthesiologists have examined PNB within the context of gross anatomy. With the advent of ultrasound technology, PNB has gained in popularity.

Microscopic study of anatomical details can add to our understanding of how to avoid fascicular injury, thereby decreasing the complications with PNB. Recent report has suggested that ultrasound guidance during PNB does not eliminate completely needle misadventure and resulting neurologic and systemic toxicity complications.[7] This is because the consequences of intraneural needle placement and injection of local anaesthetic solutions not only differ by location of the injection, but are related to the epineurium, perineurium, or endoneurium components that form the structure of the nerve at that site.

The epineurium, perineurium, and endoneurium serve different functions with regard to nerve impulse propagation in axons, protection against infection, toxins and mechanical trauma. These three layers of connective tissue are present along the entire length of the nerve, and become thinner as the nerves branches. Fibroblasts, mast cells,[8,9] macrophages,[10] collagen fibres grouped in bundles, elastic fibres and reticulin fibres can be seen among endoneurium, perineurium and epineurium.

The elasticity provided by the elastic fibres and the woven grill-pattern arrangement of the collagen fibres allow for a slight elongation of the nerves with movement. In elderly patients, the thickness of the endoneurium, perineurium and epineurium increases due to narrowing of the

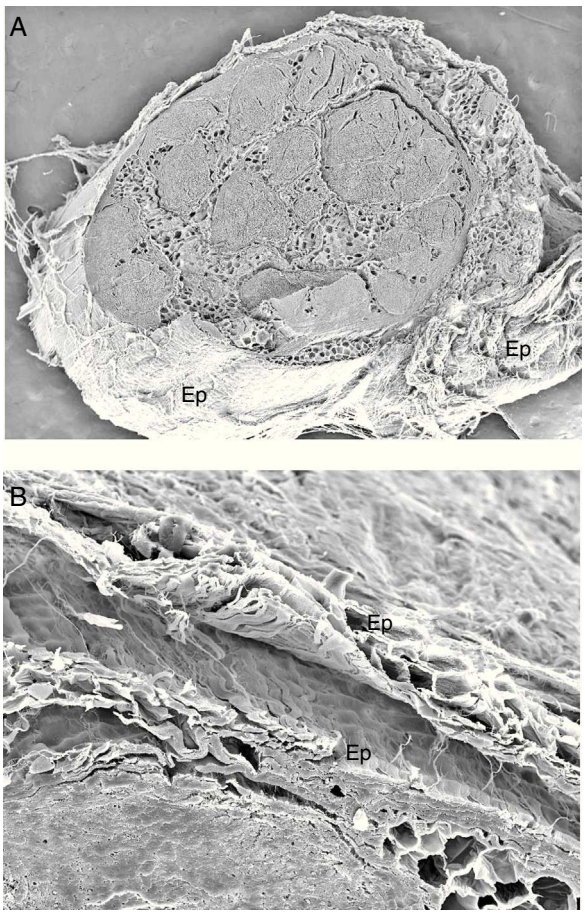

Figure 6 Epineurium in human peripheral nerve. (A and B) Tridimentional details of epineurium. SEM. Magnification: (A) 20× and (B) 180×.

arteries supplying the nerves, following endothelial proliferation and fibrosis of the tunica media.[11]

They are relevant to the performance of nerve blocks and to the occasional complications that develop. Images of peripheral nerves acquired by TEM and SEM demonstrate that the endoneurium, perineurium and epineurium are distinctly different structures, with the perineurium being the only one acting as a functional barrier.

In the peripheral nervous system, the myelinated axons are surrounded by aligned Schwann cells divided by the nodes of Ranvier (spaces between Schwann cells).[12–14] The length of a single Schwann cell is the internodal space and varies between 0.4 and 2.0 mm.[12–14] In myelinated fibres, the external diameter of Schwann cells can vary between 2 and 18 μm,[14] whereas the length of the axons can vary from a few millimetres to nearly 1 m, depending on the height of the individual.

In myelinated axons, depolarisation and repolarisation occur at the nodes of Ranvier in order to propagate the nerve impulse. This phenomenon is possible in the nodal area where the highest concentration of voltage dependent sodium channels is located.[14]

The diameter of unmyelinated axons ranges from 0.2 to 1 μm. Unmielynated axons are more or less completely submerged in longitudinal troughs formed along the Schwann cell surface. Each Schwann cell units contain more axons than some animals.[15,16]

In mammals, unmyelinated axons forms 75% in cutaneous sensory nerves and dorsal spinal roots, approximately 50% in fibres that innervates muscles,[17] and 30% of the ventral spinal roots.[18–20] Postganglionic axons are exclusively unmyelinated. Numerous unmyelinated axons are located in the preganglionic portion of nerves. Schwann cells are typical cells of the peripheral nervous system and can be found in its central portions, from the rootlets that form the nerve roots within the subarachnoid space. In humans, the process of myelination begins at the 18th week of intrauterine life, and many axons are myelinated at birth. Myelination processes decrease over the first few years of life.[21]

As a general rule the number of fascicles increases and the surface area of each fascicle decreases, in areas where the nerve branches emerge and in regions close to joints.[22–25] The nerve tend to be padded and protected by a greater amount of connective tissues in the vicinity of joints, including perineurium. This, in turn, increases the resistance of the perineurium to pressure and stretching.

In contrast, the number of fascicles decreases and the surface area of each fascicle increases, in areas away from sites of nerve subdivision and joints.[22–25] Single nerve fascicles are found enclosed by perineurium, whereas groups of fascicles appear forming bundles surrounded by varying amounts of collagen fibres. However, according to the histological technique used for sample examination these collagen fibres may be falsely identified as perineurium as it may occur with haematoxylin-eosin samples. Each bundle of nerve fascicles shares characteristic structures known as ''fascicular interconnections'' and appears enclosed by a layer of similar characteristics to epineurium. According to the anatomical location of the sample examined, there is evidence of changes in ultrastructural images from peripheral nerves. Sunderland observed that the topography of fascicles varied 23 times in just 46 mm of length.[22–25] This distribution in intraneural plexuses, may be related to embryonic development. In our study, these changes were confirmed during examination of serial cross section samples along a single peripheral nerve at intervals of few mm. Images showed axons passing through different fascicles within a bundle of nerve fascicles.

The ratio between fascicles and connective tissue in any given fascicle varies according to the portion of nerve being studied. In general, measured fascicular area fluctuates between 25% and 75% of the total nerve area[1,25,26] (Fig. 3). Since peripheral nerve blockade is performed at very different locations, our findings support the observed unpredictability in regards to both severity and extent of neural damage derived from accidental intraneural puncture and injection of local anaesthetics. Peripheral nerves of larger diameters such as the sciatic nerve contain greater amounts of fat than thinner nerves, therefore the latter are at higher risk from accidental intraneural damage.[27]

The endoneurium helps to maintain a protective environment, and plays an important role in axonal repair when cut or damaged.[28,29] It functions as an isolating device preventing disruptions in the conduction of nerve impulses among adjacent axons. It is a permeable structure without a barrier effect. Contrary to previous descriptions, portions of endoneurium examined in several samples do not exhibit

two distinctive laminas (traditionally described as the external layer in which collagen fibres are grouped and oriented longitudinal, and the internal layer where collagen fibres are loosely organized).[1,29] TEM techniques provide images of collagen fibres surrounding the conjunction of axons and Schwann cells, whereas SEM enables identification of real tunnels formed by these collagen fibres, which enclose each of the nerve axons[28] (Fig. 5). In cases where accidental intraneural injection has occurred, depending on the type of needle and the depth to which the needle is advanced within the nerve, a variable number of axons may be affected.[30–32] The tip of the needle can cause damages to axons ranging from minor injury to complete section/rupture of the nerve. The endoneurium may help to keep damaged ends of axons in correct alignment, and facilitate repair of injured axons. This is unlikely to happen when the axon is completely ruptured, but is more likely when the lesion is incomplete. The latter is more common leading to minor degree of paresthesias and may explain, in part, the low incidence of permanent neurological injury after peripheral nerve block.

The perineurium allows certain mobility to axons inside fascicles[26] and serves to maintain an intrafascicular pressure while acting as an effective physical barrier against mechanical and chemical injury. The perineurium transmits pressure to the endoneurium, which then generates intracellular pressure within the axon.[26]

Perineural cells and its specialized junctions confer selective diffusion barrier properties to the perineurium,[33–38] while protecting axons and Schwann cells from toxins, antigens, infectious agents and sudden ionic fluxes. The perineurium may allow active transport of substances by pinocytotic vesicles[35,36] and is composed mainly by cells and therefore less compliant than interfascicular tissue formed by adipocytes and collagen fibres.[39] Increases in intrafascicular pressure lead to compression of intrafascicular capillaries and nerve ischaemia.[40,41] Therefore, SEM techniques provide insights of structural details, which are central to the understanding of events leading to neurological damage after intraneural puncture and subsequent injection of local anaesthetics. As afore mentioned, tissue located within the epineurium and between fascicles (interfascicular connective tissue) is made up of loose connective tissue and adipocytes,[39] rather than more resistant collagenous tissue. Confusion exists because some authors define the epineurium as tissue within the nerve but outside the fascicles, whereas we define in this study the epineurium as the outermost dense collagen tissue enveloping nerves.[3] Interfascicular tissue lies between nerve fascicles and outside the perineurium. This interfascicular tissue is in turn surrounded by the epineurium that covers the surface of the nerve.

Most of cells occupying interfascicular tissue are similar to fat cells found outside nerves.[39] Interfascicular tissue is easily distended and permeable, and the overlaying epineurium is also elastic and friable. Larger nerves such as the sciatic are at less risk of neural damage during performance of intraneural injections due to its wider interfascicular group diameter. However, intraneural injection within interfascicular group tissue may lead to extremely prolonged anaesthetic blockade although no correlation with subsequent neurological complications has been demonstrated to date.[2]

The sciatic nerve has a complex nervous structure of interest in the performance of peripheral nerve blockade. In the sciatic nerve the interfascicular tissue ratio is greater than in nerves at the upper limb, and contains blood vessels of a larger calibre.

The epineurium is mainly composed of collagen fibres, and its thickness is proportional to the size of the nerve. The epineural layer is freely permeable and does not form a barrier (Fig. 3). Thus unlike puncture of the perineurium, damage to the epineurium seems to have no harmful effects. In dehydrated samples the epineurium is seen composed by layers and sinuously shaped along the nerve, such undulations that allow nerve stretching during movement of the limbs.

The blood–nerve barrier is formed by perineurium and intrafascicular non-fenestrated capillaries.[36] Endothelial cells from endoneural vessels are joined tightly together by specialized unions, which minimize capillary permeability.[35,36] Enclosing perineural sheath of perineurium there is axons and non-fenestrated capillaries. This sheath of perineurium is composed of large numbers of tight junctions between perineural cells,[36,42] isolating each fascicle from the interfascicular environment. After interfascicular injection, substances can reach axons either through perineural cells by diffusion or by transcellular migration of pinocytotic vesicles, which are formed at the inner side of these cells.[36] Substances in the blood stream must pass across the cytoplasm of the endothelial cells of these capillaries in order to access interfascicular tissue. Outside fascicles, substances exit through fenestrated capillaries into the interfascicular tissue, but these molecules cannot reach the axons without first passing through the perineurium described above.[43]

A needle tip piercing the epineurium will more probably encounter interfascicular adipose tissue than nerve fascicles, thus adipose tissue protects nerve fascicles from damaging effects of an advancing needle. However, this protective effect may be diminished by abrupt advancement of the needle. Similarly, it may be easier to displace a group of fascicles (fascicular bundle, Fig. 3). Injecting a solution into a group of fascicles could generate excessive pressures enough to rupture the smaller connections (fascicular connections), implying that a number of axons can also be disrupted. Although this depends on many factors, including the number of disrupted connections, the total number of axons involved, and the extent of their contribution to a particular function, it is probably advisable not to inject solutions into fascicular bundles, if at all possible.

Limitations of our study include plausible alteration of nerve samples due to vascular pathology affecting patients. Samples may have been subjected to about 10–15% reductions of their original sizes, resulting from loss of lipid vacuoles in fat tissue exposed to organic solvents such as ethanol. This has not affected ultrastructural aspects, with the exception of adipocytes loss of spherical shape. Minor variations observed in measurements among different samples are related to idiosyncrasy of patients included in the study.

In conclusions: Current practice in regional anaesthesia and ultrasound visualization of nerve structures has lead to interpretations in regards to the anatomy of human nerves, which repercussions are undoubtedly relevant in clinical

practice, and these are best ascertained by updated ultrastructural information, as is the aim of this study.

Conflict of interest

The authors declare no conflict of interest.

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