Electron Microscopy and the Expansion of Regional Anesthesia Knowledge

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We describe the findings obtained with the use of the transmission and scanning electron microscopy in the study of human meninges and peripheral nerves. We show details about morphology and distribution of the fibers inside the dural sac. We provide information about the origin of subdural space and structural organization of the arachnoid trabeculates in sleeves that wrap the nervous roots inside the subarachnoid space. We encountered natural fenestrations in the pia mater and studied ultrastructural components of the peripheral nerves coverings. A brief comment on the history of the electron microscopy and the basics of these microscopes complete the presentation of this work.

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The use of electron microscopy has broadened our knowledge about ultrastructural details related to meningeal membranes, nerve roots, and peripheral nerves. These structural findings help us to understand physiologic and pharmacologic aspects involved in regional and peripheral nerve blocks and the complications derived from such techniques in regional anesthesia.

History of Optic and Electron Microscopy

Janssen Brothers produced early works using light microscopy. During the 17th century, Malpighi, Hooke, and Leeuwenhoek observed under light microscopy living organisms immersed in water as well as different tissues. In 1886, Ernst Abbe made objectives and matched oculars that were highly corrected for spherical aberrations and also correct for 3 wavelengths of light.

Electron microscopes were developed to overcome the limitations of light microscopes that are limited by the physics of light to 500× or 1,000× magnification and a resolution of 0.2 μm. In 1931, Knoll and Ruska introduced the use of transmission electron microscopy (TEM) in Germany; similarly Oatley’s

Transmission Electron Microscopy

Electron microscopy replaces visible light (wavelength 500 nm) with a focused beam of electrons (wavelength 0.005 nm) to “image” the specimen and gain information about its ultrastructure. TEM has 3 basic components: illumination (electron gun), imaging (condenser, objective, intermediate and projector lenses), and viewing (fluorescent screen). TEM must manipulate the electron beam in a high-vacuum environment within the microscope column because air would otherwise deflect the electrons, interfering with illumination and the image-forming process. A stream of electrons is formed by an electron gun (by heating a tungsten filament) and accelerated toward the specimen using a positive electrical potential. This stream is confined and focused using objective apertures and magnetic lenses into a thin, monochromatic beam. The beam is focused onto the sample with a magnetic lens. Interactions such as absorption, interference, diffraction, and scattering occur inside the irradiated sample, affecting the electron beam. The most important interaction in TEM is scattering. Two types of electron scattering are produced: 1°, elastic, which result in deflection of the primary beam electron through a large angle, with little or no energy loss to the primary electrons (the bulk of these electrons do not contribute to information on the viewing screen) and 2° inelastic, when the primary electrons encounter electrons within the specimen; the primary electrons dislodge secondary electrons from the specimen. The effect of this interaction is that parts of the primary electron beam are removed from image formation and are replaced by secondary electrons with considerably less energy, which are scattered easily and do not reach the phosphor on the viewing screen. Inelastic scattering is the most important aspect of image formation in TEM. Electrons scattered by either mechanism do not reach the viewing screen, and, thus, a dark spot remains on the screen where the phosphor would have emitted a photon if an undeviated electron had impinged on it.

Two condenser lenses focus the stream of electrons to a small, thin, coherent beam. The first lens determines the main

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size range of the final spot that strikes the sample. The second lens changes the size of the spot on the sample, from a widely dispersed spot to a pinpoint beam. The condenser aperture (usually user selectable) restricts the electron beam, knocking out high-angle electrons (those far from the optic axis). The beam strikes the specimen and parts of it are transmitted; the transmitted portion is focused by the objective lens into an image. This image is in turn passed down the column through the intermediate and projector lenses, being progressively enlarged. The image strikes the phosphor screen and light is generated, allowing the user to see the image generated. Darker areas represent denser sites of the sample through which fewer electrons were transmitted. Thinner or less dense areas in the sample are seen as lighter because more electrons managed to pass through that portion of the sample.

**Scanning Electron Microscopy**

This microscope provides information about the shape, size, and arrangement of the particles that are lying on the surface of the sample. An electron gun produces a stream of monochromatic electrons; the first condenser lens condenses the stream, and the second condenser lens gives way to a thin, tight, coherent electron beam. A set of coils then scans or sweeps the beam in a grid fashion (like a television), dwelling on points for a period of time determined by the scan speed (within the microsecond range). The objective is to focus the scanning beam onto the part of the specimen desired; when the beam strikes the sample (and dwells for a few microseconds), interactions occur inside and are detected with various instruments. Before the beam moves to its next dwell point, these instruments count the number of interactions and display a pixel on a cathode ray tube whose intensity is determined by this number (the more reactions, the brighter the pixel). This process is repeated until the grid scan is finished and then repeated; the entire pattern can be scanned 30 times per second. Samples must be coated with a heavy metal microfilm. Samples observed under SEM do not require being ultrathin because the images are obtained from the surface of the sample. The power of resolution of SEM is in the range of 10 nm; therefore, the images produced by SEM compared with those produced by light microscopy with equal augmentation are 10 times clearer, giving a perfectly defined tridimensional view of the sample’s surface.

**Preparation of Biologic Samples**

**Biologic Samples Need Specific Preparation Depending on the Type of Electron Microscope Used**

**Transmission electron microscopy.** Samples are fixed for 4 hours in a solution of glutaraldehyde 2.5% and a buffer phosphate solution to a pH of 7.2 to 7.3. Postfixation of the samples with a solution of 1% osmium tetroxide during 1 hour was followed by coating with gold microfilm. Samples observed under SEM do not need ultrathin cutting; they undergo dehydration, which enables the samples to be observed under TEM. The electron beam does not cross samples thicker than 100 nm; therefore, ultramicrotomes are needed to obtain such thin cuts. The knives used in the ultramicrotome are made of glass or diamond; the samples must be cut under light microscopy. To enhance the image contrast, the slides are stained with uranyl acetate and later mounted on a cooper grill as the electron beam crosses the orifices of the grill. Samples to observed under SEM do not need ultrathin cutting; they undergo dehydration, followed by coating with gold microfilm.

**Insights in Regional Anesthesia Related to Electron Microscopy**

Electron microscopy allowed us to study meningeal membranes and peripheral nerves from samples of human cadavers immediately after death. We observed the ultrastructural details of the dura mater, the laminar portion of the arachnoid internally covering the dural sac, and its trabecular components; we also observed cellular organization of the subdural compartment as well as the pia mater. In relation to peripheral nerves, we obtained samples from sciatic nerve belonging to the upper end of the popliteal fossa; we observed detail of myelinic and nonmyelinic axon and epineurium, perineurum, and endo-
neurum; the blood-brain barrier and the intraneural adipose
distribution were also studied.

Traditional knowledge about the structure of the meningeal membranes was brought about by light microscopy. SEM al-
lowed us to obtain 3-dimentional views from samples up to 2 cm in size, giving in this way an overall image of the surface structural details. In simple terms, SEM acts as a highly magni-
ified lens providing views from the samples' surface while avoiding image distortion as we focus sites located at different depths within the surface; this facilitates our observations. TEM is
needed to complement the information obtained in the SEM.

TEM provides information about internal structures from sam-
pies observed under SEM.

Dura Mater

The spinal dura mater has a cylindrical laminar layer with a
thickness of 1 mm. At lumbar level its thickness is reduced to
320 μm; this measure varies in the anteroposterior and lateral
portions of the dural sac. The dural laminar layer is made of
about 80 well-defined concentric laminas, and each lamina is in
turn formed by 8 to 12 thin laminar subunits (Fig 1). Dural
collagen fibers with a thickness of 0.1 μm and a smooth surface
are the constituents of these subunits, oriented in different
directions within the subunit main plane. Dural collagen fibers
orientation is not radial within the dural sac but tangential in

Fig 1. Dura mater. Partial thickness of dura mater. Detail of
dural laminas. SEM, original magnification 4,000×. (Re-
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Fig 2. Dura mater. Epidural surface of dural sac. Detail of
collagen fibers. SEM, original magnification 1,000×. (Re-
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Fig 3. Dura mater. Epidural surface of dural sac. Detail of
elastic fibers. SEM, original magnification 7,000×. (Reprinted
with permission.

Fig 4. Origin of subdural space. The break of the neurothelial
cells allows the separation of the laminar arachnoid to the
dura mater. This gives origin to the space subdural. SEM,
original magnification 180×. (Reprinted with permission.

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relation to the spinal main axis, and there is not overlapping between collagen fibers from different laminas² (Fig 2). Three fiber orientation patterns have been defined: type 1, a lax weaving of easily individualized fibers distributed in different directions; type 2, fibers forming bundles in a given direction, each bundle in turn consisting of dozens of fibers separated by ground substance; and type 3, these are type 2 fibers that merge to form compact plaques of similar width.³⁴

We found elastic fibers scarcely distributed among collagen fibers, with a thickness of 2 µm and a rough surface³⁴ (Fig 3). In posterior portions of the dural sac, the elastic fibers are mainly oriented vertically; in lateral portions of the dural sac, these elastic fibers orientation is more oblique, different to the orientation seen in elastic fibers found in dural sleeves surrounding nerve roots, where they are placed more horizontal in relation to the spinal main axis. In these areas, the dura is thinner, with a thickness of about 80 µm.³ Under SEM, macrophages and fibroblasts were not identified.

Dura mater occupies 90% of the external thickness of the dural sac; its main component is collagen fibers that contribute to the mechanical support of the dural sac. Spaces were seen between collagen fibers usually filled by an amorphous substance that had been eliminated by agents such as phosphates and acetone during the preparation process. Hydrophilic substances follow the path throughout amorphous substance to cross the dura mater. TEM showed structural details of collagen
fibers such as the presence of striations located at a constant distance of about 67 Åmstrong.

Transversal cuts from samples containing collagen fibers were observed and confirmed that the traditional thought about collagen fibers being oriented parallel and longitudinal within a tangential plane is not true; this finding was also confirmed by SEM. However, TEM could not show precise details of each thin dural lamina. We identified, under TEM, fibroblasts parallel to dural laminas and macrophages present between collagen fibers. These fibroblasts are flattened and measure about 1 μm; their nuclei contain finely dispersed chromatin and their cyto-

Fig 9. Dural lesion produced by Quincke needle. SEM, original magnification 200×. (Reprinted with permission.10)

Fig 10. Dural lesion. Details of figure 9. SEM, original magnification 5,000×. (Reprinted with permission.10)

Fig 11. Dural lesion produced by Whitacre needle. SEM, original magnification 300×. (Reprinted with permission.10)

Fig 12. Peripheral nerve. Details of fascicles and the intraneural adipose cells. SEM, original magnification 50×. (Reprinted with permission.11)

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plasm is rich in organelles (mainly mitochondria), with a scarce granular endoplasmic reticulum, free ribosomes, and a number of membrane involutions suggestive of pynocitosis or exocitic processes.

The vast majority of information given by textbooks was based in data obtained with light microscopy, but images under light microscopy can easily be misinterpreted because it only allows the observation of fiber distribution coinciding with the sample's cut orientation. SEM and TEM have obvious advantages because they provide extensive 3-dimensional images that help with the analysis and evaluation of structural details from views obtained.

Arachnoid

With the use of MEB, we could see that the arachnoid has a laminar layer covering the inner side of the dural sac and occupies 10% of arachnoid internal thickness\(^5,6\) (Fig 4). The arachnoid membrane has also a trabecular layer placed between the laminar portion of the arachnoid and pia mater. Arachnoid's laminar portion contributes to the dural sac's semipermeability properties. Different cellular components are not identified, but it is possible to measure the thickness of the arachnoid mater.\(^5,6\)

The arachnoid trabecular layer encloses the structures that traverse the subarachnoid space. In this way, nerve roots are not free within the dural sac but surrounded by arachnoid structures known as arachnoid sheaths\(^7\) (Figs 5-7). Under MEB, we measured the thickness and observed the structure of these arachnoid sheaths that help in keeping nerve roots structurally organized within the dural sac, limiting positional shifts with respect to neighboring roots during spinal movement.

TEM showed the arachnoid laminar layer, which covers the inner side of the subdural compartment. The arachnoid laminar layer has 2 components: barrier lamina and reticular lamina. The barrier lamina has a thickness of 5 \(\mu\)m; its cellular plane looks like epithelial tissue, less flattened than neurothelial cells that occupy the subdural compartment. This lamina is clearly distinct to reticular lamina because of the presence of compact junctions between its cells. A basal membrane separates both lamina. The reticular lamina has a thickness between 10 and 20 \(\mu\)m and is formed by irregularly interleaving cells, alternating with an increased amount of collagen fibers and intercellular lacunar spaces of variable size filled with amorphous material.

The arachnoid trabecular layer is made of several trabeculas; each of them is composed of bundles of collagen fibers and covered by flat cells that are similar to those found in the barrier lamina. We found macrophages around this type of cells.

Subdural Compartment and Subdural Space

Observed under SEM, the subdural compartment has neurothelial cells located between the last dural lamina and the barrier arachnoid lamina. Tearing within the thickness of the subdural compartment may open up the subdural space; areas between neurothelial cells are most susceptible to tearing and cellular fragments may be seen outside torn neurothelial cells. Low-cohesion forces between neurothelial cells facilitates the widening of a minimal fissure to give up a real subdural space; other smaller fissures can be seen parallel to the main space and are called secondary subdural spaces (Fig 4).

We showed, under MEB, different types of cells, structural details of neurothelial cell, and arachnoid cells. It is relevant to mention the lack of collagen fibers surrounding neurothelial cells. These collagen fibers were abundant around arachnoid

![Fig 13. Human myelinic axon surrounded by Schwann's cells. TEM, original magnification 12,000×. (Reprinted with permission.)](image1)

![Fig 14. Human myelinic axon. Details of myelin. TEM, original magnification 85,000×. (Reprinted with permission.)](image2)
cells and in the dural laminas. Arachnoid cells presented specialized junctions differently to neurothelial cells that did not have them. We could verify that it is the tearing of neurothelial cells that gives origin to a real subdural space that will not be present otherwise. Therefore, the term virtual subdural space is incorrect.5,6

Pia Mater

Under MEB, pial cells constitute a cellular plane with fenestrations similar in sizes to pial cells.8 Through these fenestrations, we could see different components of the subdural compartment8 (Fig 8). This has a variable thickness, depending on the medular area or spinal root observed, and collagen fibers are its main constituent. Under TEM, we identified pial cells, its organelles, and a number of pynocitic vesicles inside the cytoplasm; we also saw a basal membrane separating the subpial compartment from glial tissue as well as Schwann cells and axons.

Dural Lesions Induced by Quincke- and Whitacre-Type Beveled Needle

Fifteen minutes after withdrawing a spinal needle from the subarachnoid space, we observed the damage done by the needle to the dural sac (dura mater and arachnoid). Orifices piercing the dural lamina were seen on the external surface of the dural sac in contact with the epidural space; on the internal surface, the lesion caused to the arachnoid can be bigger than that in the dura mater because of arachnoid different elastic properties, which take longer to return to its initial position, reducing in this way the size of the orifice.

We did not find significant differences in cross-sectional areas of the punctures produced by 25G-Quincke or 25G-Whitacre spinal needles.9 We also compared the damage caused to the dura-arachnoid when introducing a Quincke needle with the bevel parallel to the spinal axis with the damage produced by introducing the bevel perpendicular to the spinal axis; we did not find significant differences. After 15 minutes, the dura-arachnoid lesion is reduced by about 82% to 87%.10 Differences in these lesions morphology were observed (Figs 9-11). However, although Whitacre needles produced coarse lesions with significant destruction of dural fibers, Quincke needles typically produced a U-shaped or flap-like lesion resembling the open lid of a tin can regardless of the needle tip direction.

Peripheral Nerves

SEM allows the elaboration of intraneural topograms giving data about shape and distribution of neural fascicles belonging to peripheral nerves and at different sites along them. With SEM, we observed details about epineuro, perineuro, endoneuro, as well as intraneural vessels and adipose tissue distribution within the neural fascicles. The sciatric nerve in the superior angle of the popliteal fossa is seen as a single branch with light microscopy, but SEM showed 2 branches held together by connective tissue. The internal branch is bigger and corresponds to the tibial nerve, whereas the smaller branch is the common peroneal nerve; the connective tissue is filled mainly with adipocytes. These adipocytes were of similar sizes, about 40 μm and appeared empty as fat was eliminated during the preparation process, losing their natural shape11 (Fig 12). Adipose tissue is distributed within the epineurium, covering single nerve fascicles or wrapping around groups of fascicles.

Blood-Nerve Barrier in Peripheral Nerves

By means of TEM, we study the perineurium enrolling nerve fascicles and their capillary vessels. The perineurium comprises about 8 to 15 concentric layers of cells interposed with collagen fibers enclosing every fascicle.11 Each of these layers has a single cellular layer. A larger number of layers was found in the epineurium surrounding bigger fascicles. Flat perineural cells are smooth and showed specialized junctions (zonula occludens, hemidesmosomas) contributing to a barrier effect. Perineural cells have an internal membranous system capable of producing several pynocitic vesicles.12 The endoneuro appears as a thin tubular structure wrapping round Schwann’s cells and around each of the capillaries found inside the fascicles. In the thickness of the endoneuro, there were no cells but instead collagen fibers orientated in a longitudinal direction.

The majority of the intra-fascicular vessels were capillaries covered with endoneuro. We did not see arterioles inside the fascicles, and most of them were found within compartments outside the fascicles. We observed mielinic axons of different diameters surrounded by Schwann’s cells (Figs 13 and 14) and groups 6 to 14 nonmyelinic axones surrounded partially by a single Schwann’s cell located between the axones.

References