Immunohistochemical and electron microscopy aspects of the nerve structures from the dental pulp

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Abstract
In this study, we have done an immunohistochemical and an electron microscopy examination of normal and inflamed human dental pulp specimens in order to evaluate the morphological aspects of the nerve structures from the dental pulp. The S100 protein immunohistochemical marking allowed us to observe the trajectory of the pulp nervous structures, which appear as continuous bands of high intensity at radicular level, coronary branch out and some branches cross the odontoblastic layer and penetrate in predentin along the dentinal tubules. It appears that not only the nerve structures are positive S100 protein but also macrophages or dendritic cells. The electron microscopic part presents the ultrastructure details of the nervous structures observed on the samples from normal and inflamed pulp conjunctive tissues. Even in acute pulpitis no ultrastructural changes occur in the nerve fibers, prolonged exposure to noxious factors may lead to changes like nerve sprouting.

Keywords: dental pulp, nerve structures, immunohistochemistry, electron microscopy.

\section*{Introduction}
The dental pulp is a common site of disease and this disease is typically associated with pain. In fact, the application of various stimuli to either exposed dentin or to pulp tissue generally produces the sensation of pain [1].

The nerve fiber density within the human dental pulp is quite impressive and multiple studies have characterized these structures. Teeth have unusual neural features such as dense polymodal nociceptive sensory innervation of coronal dentin, pulp, and vasculature; sparse autonomic innervation and sensory nerve involvement in dentinal fluid dynamics, pulpal blood flow regulation, protective reflexes, to preserve dental tissues, and dental wound healing [2].

In this study, we have done an immunohistochemical and an electron microscopy examination of normal and inflamed human dental pulp specimens in order to evaluate the morphological aspects of the nerve structures from the dental pulp.

\section*{Materials and Methods}
For the realization of this study, we used 20 samples of pulp tissue divided into two groups.

The first study group was represented by samples of pulp tissue collected from teeth without clinical symptoms of pulpitis. The collecting was made following the pulpectomies made for prosthetic reasons in eight cases or after the teeth extraction, for two ectopic wisdom molars.

The second study group was represented by 10 samples of pulp tissue collected either directly from the pulp chamber following the pulpectomies made for therapeutic reasons, in six cases, either after the extraction of the teeth that presented also irreversible periodontal lesions (e.g., tooth mobility grade 2–3), in the other four cases.

The pulpectomies were made under local anesthesia with 3% Mepivacaine, without vasoconstrictor. The collecting of the pulp tissue subjected to the study was preceded by a less brutal opening of the pulp chamber with the aid of the micromotor and of spherical burs of adequate dimensions for the teeth on which the operation has been performed. The proper collecting was made from the pulp chamber with the aid of well-sharpened dental excavators of adequate dimensions; they were used to cut off, systematically, the pulp tissue from the pulp chamber walls, together with the odontoblastic layer.

When the clinical situation imposed the tooth extraction, immediately after the tooth was pulled out, we made with the aid of a dental turbine, under water-cooling, two longitudinal ditches on opposite sides of the tooth. With a dental elevator applied in one of the two ditches, we separated the two fragments. The exposed pulp tissue exposed in this way was drawn with a dental excavator, together with the odontoblastic layer.

For the immunohistochemical study, the pieces were passed into a fixing solution of 10% neutral formalin, for 24 hours. Eventually, the pieces were treated following the classical histological technique for including into paraffin, technique that allowed us to slice serial 5 μm
thick sections. The sections were placed on histological blades treated with poly-L-Lysine (Sigma-Aldrich, Munich, Germany). The immunohistochemical method used in this study was the Avidin–Biotin Complex (ABC) three-stage indirect method. In this study, we monitored the answer of the connective pulp tissue for the S100 protein antibody (Dako, microwaves pretreatment, 650 W, citrate buffer pH 6, dilution 1:100).

In collecting the fragments for the electron microscopy study, we took into account two conditions imposed by the electron microscopy study:

- the tissue for study must get into the fixation (2.5% glutaraldehyde in 0.15 M sodium phosphate buffer, pH 7.2) in maximum 2–3 minutes after the irrigation with blood ceased;
- the fragments of tissue collected must not be over the volume of 1 mm³. If the collected piece was bigger, it was cut in cubes of maximum 1 mm³, by means of two new razor blades, joined and moved parallel so not to press the tissue.

The samples collected this way were included in Vestopal 310 resin, followed the classic stages necessary for the preparation of the human histological material having in view the study on the electron microscope with transmission. The examination was done by means of a JEOL JEM1010 electron microscope with CCD camera, within the Center for Electron Microscopy of the “Babeș–Bolyai” University, Cluj-Napoca, Romania. The analysis of the images and the interpretation of the results were done in cooperation with a specialist in ultrastructural studies.

**Results**

The microscopic aspect of the dental pulp revealed a peripheral and a central zone. The peripheral one contains an odontoblastic layer, a free-cell zone known as the Weill zone, and a zone rich in cells with an intermediate form between fibroblasts and odontoblasts known as Hohl cells (Figure 1). The central pulp presents a light connective tissue, with fibroblasts and fibrocytes (Figure 2).

The dental pulp nerve fibers penetrate through the apex together with the blood vessels. We frequently noticed on the studied preparations nerve trunks associated with blood vessels. The main nerve trunks branches then progressively on the face of the walls pulp. They arrive in the under and periodontoblastic area, where they form a plexus which was first described by Rashkov.

At the immunohistochemical study on samples of pulp tissue collected from teeth without clinical symptoms of pulpitis, the positive structures for the S100 antibody appear in the radicular pulp tissue more like continuous structures with high intensity (Figure 3), as a band that pursues the trajectory of blood vessels (Figure 4), while in the coronary pulp they are repeatedly branching resulting positive areas with smaller thickness, which can present interruptions. A series of S100 positive structures cross the odontoblastic layer and penetrate in the predentin along the dentinal tubules.

On samples of pulp tissue following the pulpectomies made for therapeutic reasons, we noticed an increase of the density of immunoreactive nerve fibers (Figures 5 and 6). It appears that not only the nerve structures are positive for the S100 protein but also a series of cells involved in the evolution of the inflammatory process (data not showed).

The pulp sensitive fibers penetrate through the apical foramen and follow approximately the blood vessels trajectory. At the electron microscopy study, we noticed the myelin fibers with a diameter varying from 2 to 20 µm. They consist of neuronal extensions that have myelin sheaths and sheaths of Schwann cells, but are devoid of connective perineuronal sheaths (Figure 7). These nerve extensions contain long filaments of 150 Å diameter, parallel to each other and to the longitudinal axis of the fiber, many neurotubules, mitochondria and vesicles of lysosomal type. The myelin sheath has a thickness of 0.5 to 1 µm. It presents a lamellar structure and it is composed of alternate layers of lipids and proteins. It is built up by the proliferation of the plasma membrane of Schwann cells (Figure 8). The myelin sheath is not continuous but is interrupted by the nodules of Ranvier. It may present irregularities that seem to be characteristic of the pulp innervation (Figure 9). The Schwann cells surround 1–10 nerve extensions.

The unmyelinated fibers are located in grooves of the cell membrane. These are thinner fibers than the
myelinated ones with a diameter of 2 μm. The Schwann cells are separated from the surrounding tissue by a basement membrane (Figure 10). In the terminal branches the Schwann cell envelope is missing, then disappears also the myelin sheath. They end up in the under and periodontoblastic area, where it forms the Rashkov plexus. At this level, nerve extensions do not present between them specialized junctions of synaptic type and the terminal ends do not present specific structures, but are free endings.

We did not found important differences between the two study groups at the electron microscopy study, except one case represented by a tooth with periodontal lesions where we noticed the presence of sprouts on a few nerve fibers and one Ranvier node with an atypical form.

Figure 3 – Positive reaction of the pulp nerve fibers for the S100 protein, ×400.

Figure 4 – Positive structures for the S100 protein adjacent to a pulp blood vessel, ×400.

Figure 5 – Pulp nerve fibers transversely sectioned, positive for the S100 protein, ×200.

Figure 6 – Detail of the previous image with the presence of positive nerve fibers for the S100 protein, ×400.

Figure 7 – Transversely sectioned nerve fibers. It highlights the myelin sheaths, the nucleus of a Schwann cell, neurotubules and neurofilaments. Scale bar = 5 μm.

Figure 8 – Transversely and longitudinally sectioned nerve fibers with myelin sheaths highlighting. Scale bar = 1 μm.
Discussion

The dental pulp is a complex connective tissue of mesenchymal origin developed from the dental papilla [3], composed of cells (fibroblasts, immune cells, odontoblasts) fundamental substance, connective fibers, nerves, blood and lymphatic vessels [4]. The sensory innervation of the dental pulp is made by axons originating from the trigeminal ganglia [5].

The structural elements of the dental pulp undergo changes also in normal conditions but especially under pathological conditions (trauma, carious lesions, pulpitis, periodontitis) [6]. Thus, decreased sensitivity of periodontally diseased teeth may be related to the degeneration of myelinated nerve fibers in the pulp [7].

The S100 protein is a marker especially of nervous tissue cells: glial cells, neurons, Schwann cells. The immunohistochemical reactivity study of this protein in the dental pulp tissue allowed an analysis of the nervous structures disposition at this level. Thus, if in the radicular pulp tissue the S100-positive structures appear more as continuous structures, with great intensity, as a band that follows the trajectory of blood vessels, in the coronal pulp they are repeatedly branching resulting positive zone with a reduced thickness. Of the two subunits of the S100 protein, just the beta form is present in the axons from the dental pulp and from predentin, as well as in the Schwann cells [8].

Although some studies have found positive responses to S100 protein only at the level of the nervous structures from dental pulp [9], we still met this protein also in other pulp cell populations and especially in the pulp inflammation. In fact, even since 1989, Lombardi and Castellucci [10] showed a strong positive reaction to the S100 protein for the pulp macrophages together with Schwann cells, and other studies have shown the positivity also for the dendritic cells [11].

In our study, we found on the samples of pulp tissue following the pulpectomies made for therapeutic reasons an increase of the density of immunoreactive nerve fibers. In our opinion, this increase of the density of the positive pulp structures for the S100 protein during inflammatory processes is due specifically to the fact that not only the nerve structures are positive for S100 protein, but also macrophages or dendritic cells.

If until now we could talk about the use of the S100 protein as a marker for dental pulp innervation, recent researches, also supported by the observations from the present study carried out on inflamed pulp tissue samples and which have noticed an increase in the density of immunoreactive nerve fibers in these cases, support the idea of using S100 protein also for determining the extent of the pulp inflammation and even the possibility of use in achieving alternative ways of the pulp biologic therapy in order to reduce the inflammatory process [12].

The dental trigeminal axon sizes at their entry into the tooth include C-fibers, small and large A-delta fibers, and some A-beta fibers [13]. All types then branch many times, and most end in the crown as free nerve endings along blood vessels, in the under odontoblastic plexus, in the odontoblast layer, in predentin, or in the inner 0.1–0.2 mm of coronal dentinal tubules [14].

In our electron microscopy study, we noticed the pulp sensitive fibers that penetrate through the apical foramen and follow approximately the blood vessels trajectory. The myelinated fibers have a diameter varying from 2 to 20 µm. However, the myelinated axons undergo extensive morphologic changes during their course from the radicular to the peripheral pulp [15]. The unmyelinated fibers are located in grooves of the cell membrane. These are thinner fibers than the myelinated ones with a diameter of 2 µm. A progressive loss of myelin was seen within the tooth also in rat studies since the proportion of unmyelinated axons is greater at more coronal locations than seen near the root apex in rat molars [16].

The human dental pulp is richly innervated by unmyelinated nerve fibers. Our results and the results of others [17] suggest that many of these unmyelinated nerve fibers actually originate from myelinated fibers and therefore much of the pain associated with toothache may actually involve the activation of larger diameter neurons. The thinning of fibers due to myelin loss appears as a prominent feature of pulp afferents that may represent a unique phenotype [1].

The nerve endings are found in close relation with the odontoblasts. Some of them penetrate also in dentin, through the dentinal tubules, where they twist around the...
odontoblast extensions [18]. The unique spatial situation of odontoblasts, ciliated cells in close relationship with nerve terminals, suggests that they could play a pivotal role in the transduction of sensory events occurring within the dentin tissue [19].

We also noticed vasomotor fibers in close relation with the smooth muscle fibers from the vessel walls. They control and regulate the blood flow. These fibers are devoid of a myelin sheath, they are surrounded only by the Schwann cell sheath. They are located along the arterioles and the meta-arterioles, and at the pre-capillaries sphincters. At the periphery of the pulp, the terminal unmyelinated fibers lose the Schwann cell sheath and they are noticed near the under-odontoblastic capillaries loops [20].

In our electron microscopy study, we did not found important differences between the two study groups, except the presence of sprouts on a few nerve fibers and one Ranvier nodule with an atypical form. However, a number of studies showed that the nerve fibers have important effects on pulp blood flow and inflammation, while their sprouting and cytochemical changes after tooth injury are in response to altered pulp cytochemistry [21]. In addition to the nociceptive alarm signaling, the intradental sensory axons may play a regulatory role in the maintenance and repair of the pulpodental complex [22].

There are evidences in support of interactions between the sympathetic nervous system and dental inflammation. The complexity of the neural regulation of teeth is demonstrated by the different actions of sensory nerve fibers on blood flow in pulp. In a study on inflamed teeth, the proportion of the A-delta fibers responding to dentinal stimulation was significantly higher than in uninflamed control teeth, and the difference was most pronounced in A-delta fibers [23]. Such changes could be due to nerve sprouting or to activation or sensitization of normally unresponsive nerve endings of individual axons [14]. Results from other studies [24] consistently showed a prominent demyelinating reaction of axons within the pulpitis samples that resulted in the increased incidence of atypical nodal forms. Sprouting of sympathetic nerve fibers occurs in chronically inflamed dental pulp, and neural imbalance caused by unilateral sympathectomy recruits immunoglobulin-producing cells to the dental pulp [25].

In our electron microscopy study, the samples from the second group, the therapeutic one, have originated from acute pulpitis. In the light of these literature data, we may assume that morphological changes in the dental pulp nervous structures occur after a prolonged exposure of them to an inflammatory process, those changes being able to be visible mostly in preparations from chronic pulpitis.

Conclusions

The S100 protein immunohistochemical marking allowed us to observe the trajectory of the pulp nervous structures, especially in the absence of inflammation, while in the samples from cases with pulp inflammation we found an increase of the density of the pulp structures positives for the S100 protein. It appears that not only the nerve structures are positive for the S100 protein but also macrophages or dendritic cells. We noticed at the electron microscopic study thinner unmyelinated fibers, located in grooves of the cell membrane, and myelinated fibers with a diameter ranging from 2 to 20 µm, with myelin sheaths that may present characteristic crazing and interrupted by the nodules of Ranvier, sometimes with atypical shapes on samples from cases with pulp inflammation.

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