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ORIGINAL ARTICLE

IMMUNOHISTOCHEMICAL VISUALIZATION OF HUMAN DENTAL PULP NEURAL DEVELOPMENT BY DETECTION OF NF-H AND NESTIN

Visualización inmunohistoquímica del desarrollo neural de la pulpa dental humana mediante la detección de NF-H y nestina

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ABSTRACT

Objective: To descriptively assess the expression of Neurofilament heavy (NFH) and Nestin in the dental pulp (DP).

Material and Methods: Fifteen human teeth were extracted and classified according to three Moorrees tooth development stages: initial root formation (Ri), root length ½ (R1/2), and root length complete (Rc). Immunohistochemical assays were performed for NFH and Nestin and analyzed under light microscopy. Images for each antibody immunoexpression in tissue sections from each stage of root development were qualitatively analyzed to evaluate the spatial distribution for each antibody.

Results: Paraffin-embedded tooth sections stained with hematoxylin and eosin showed an apical cell-rich zone between the DP and the apical papilla. NFH was expressed in the core of the dental pulp as bundles of nerve fibers and sprouts. NFH immunostaining is closely associated with arteries and capillaries in all the developmental stages samples. Neuroepithelial stem cell protein (Nestin) was highly expressed in differentiated odontoblasts in the predentin odontoblast and odontoblast cell processes, indicating a reservoir of newly differentiated odontoblast-like cells.

Conclusions: Nestin is a crucial antibody in the early stages of dental pulp tissue development, and NFH remains in the core of mature dental pulp suggesting its role in the tissue's homeostasis. Successful tissue regeneration depends directly on forming a functional vascular network and innervation. Therefore, the full comprehension of the biological features provides valuable insights into the complex challenge of regenerating dental pulp innervation.

Keywords: *Dental pulp; Neurogenesis; Nestin; Neurofilament proteins; Nerve fibers; Stem cells*

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RESUMEN

Objetivo: Evaluar descriptivamente la expresión de neurofilamento pesado (NFH) y nestina en la pulpa dental (PD).

Material y métodos: Se extrajeron quince dientes humanos y se clasificaron según tres estadios de desarrollo dental de Moorerees: formación radicular inicial (Ri), longitud radicular ½ (R1/2) y longitud radicular completa (Rc). Se realizaron ensayos inmunohistoquímicos para NFH y nestina, los cuales se analizaron mediante microscopía óptica. Las imágenes de la inmunoexpresión de cada anticuerpo en cortes de tejido de cada estadio de desarrollo radicular se analizaron cualitativamente para evaluar la distribución espacial de cada anticuerpo.

Resultados: Los cortes de dientes incluidos en parafina y teñidos con hematoxilina y eosina mostraron una zona apical rica en células entre la PD y la papila apical. El NFH se expresó en el núcleo de la pulpa dental en forma de haces de fibras nerviosas y brotes. La inmunotinción de NFH se asoció estrechamente con arterias y capilares en todas las muestras de los estadios de desarrollo. La proteína de células madre neuroepiteliales (nestina) se expresó en gran medida en odontoblastos diferenciados, tanto en las prolongaciones de los odontoblastos predentinarios como en los odontoblastos, lo que indica la existencia de una reserva de células odontoblastoides recién diferenciadas.

Conclusiones: La nestina es un anticuerpo crucial en las primeras etapas del desarrollo del tejido pulpar dental, y la NFH permanece en el núcleo de la pulpa dental madura, lo que sugiere su función en la homeostasis del tejido. La regeneración tisular exitosa depende directamente de la formación de una red vascular e inervación funcionales. Por lo tanto, la comprensión integral de las características biológicas proporciona información valiosa sobre el complejo desafío de regenerar la inervación de la pulpa dental.

Palabras clave: *Pulpa dental; Neurogénesis; Nestina; Proteínas de neurofilamentos; Fibras nerviosas; Células madres*

INTRODUCTION

The dental pulp (DP) is a specialized connective tissue, highly vascularized and innervated, and presents specialized physiological functions.[11] DP is described as a complex system in which odontoblasts, sensory nerves and Schwann cells, associated with immune and vascular components, form a multicellular interface related with the sensory protection, tissue homeostasis, defense and repair processes of the tooth, surrounded by mineralized tissue that protects them.[2,3]

Human dental pulp is innervated by trigeminal sensory afferents characterized as myelinated and unmyelinated axons.[3] The sensory nerve fibers enter the pulp cavity with the blood vessels through the apical foramen. Those are mixed fibers including sympathetic fibers from sympathetic nodes and the sensory nerve fibers myelinated A-delta and unmyelinated C fibers.[4]

Pulp tissue present a high nerve fiber density that confers unusual neural features: dense polymodal nociceptive sensory innervation, that participate in inflammation, immunity and angiogenesis, limited autonomic innervation and sensory involvement in dentinal tubules fluid dynamic, regulation of the blood flow, etc.[2]

During human tooth development, the pulp

innervation is modulated by different molecular mechanisms, non-myelinated axons continuously sprout as they approach the dentinpulp interface, it has been stated that innervation is a key component of the process. In addition to the sensory function related to the stimuli response to protect the tooth, innervation plays a critical role in pulp homeostasis.[3–5]

To achieve functional pulp regeneration, restoring the innervation is key, therefore, the understanding of the innervation process in the DP is essential to establish regenerative biological approaches in correlation with the development of the natural tissue. On the other hand, the neuroepithelial stem cell protein commonly known as Nestin and the neurofilament heavy chain protein are neural signaling expression markers classified as intermediate filaments that constitute the major component of the neural cytoskeleton.[6]

Different types of intermediate filaments have been described, in the peripheral nervous system, are heteropolymers composed of subunits or type IV intermediate filament proteins: neurofilament light (NF-L), neurofilament medium (NF-M), neurofilament heavy (NF-H) and peripherin. NFs make up the main structure of axons and dendrites, are involved in morphogenesis of neurons, the maintenance of neuronal caliber, and are found in neurons, peripheral nerves and sympathetic ganglion cells.[7,8]

Nestin is a type VI intermediate filament, expressed in a cell-type specific manner, initially described in neural stem cells in the central nervous system and subsequently observed in a variety of tissues and progenitor cells, among them, in the dental pulp expressed in regenerative peripheral neurons and blood capillaries.[6,9,10]

In this study, we performed an immunohistochemical examination of dental pulp in different stages of root development in order to descriptively assess the expression of Neurofilament heavy (NFH) and Nestin in the dental pulp (DP).

MATERIALS AND METHODS

Ethical statement

This research protocol was reviewed and approved by the Ethics Committee of the University of Costa Rica, San José, San Pedro, Costa Rica (Protocol Number: VR-467-2018). All procedures adhered to the ethical principles outlined in the Declaration of Helsinki (1975).

Sample collection

Fifteen healthy, young human permanent teeth (age range 6-15 years)

Fifteen healthy, young human permanent teeth (age range 6-15 years) were collected between 2022 and 2023 following extraction for reasons unrelated to this study.

Inclusion and Exclusion Criteria

The inclusion criteria were:

(1) single-rooted morphology;

(2) sound structure, defined as intact enamel and dentin without macroscopic evidence of structural compromise;

and (3) root development categorized as initial root formation (Ri), root length at one-half completion (R1/2), or root length complete (Rc) according to the Moorrees morphological classification of dental development.

Teeth were excluded if they presented any of the following criteria:

(1) macroscopic evidence of dental caries; (2) resorptive defects (internal or external); or

(3) complex root anatomy deviating from a typical single-rooted morphology (e.g., fused roots, gemination).

These criteria were implemented to ensure a homogeneous sample representing specific stages of root development (Ri, R1/2, and Rc) in healthy teeth collected during the specified timeframe.

Specimen Processing

Extracted teeth were immediately fixed in 10% neutral buffered formalin (05310, Laboratorios Químicos ARVI S.A, Costa Rica) at a 25:1 volume ratio for 48 hours. Following fixation, specimens underwent decalcification in rapid hydrochloric acid Shandon TBD-1 Decalcifier (6764001, 6764001,Thermo Scientific, USA) at a 20:1 volume ratio until complete decalcification was confirmed via physical probing (72-96 hours). Decalcified samples were then washed in running water for 1 hour.

Longitudinal sections were prepared to expose the pulp tissue. Subsequently, the specimens were manually dehydrated in a graded ethanol series at room temperature: 70% for 24 hours, 80% for 1 hour, 95% for three changes of 2 hours each, and 100% overnight followed by two changes of 1 hour each (Laboratorios Químicos ARVI S.A, Costa Rica). Clearing was performed in xylene (three changes of 1 hour each (X0056 Diapath, Italy), followed by embedding in paraffin wax (Diawax, Diapath, Italy) at 56°C-58°C (two changes of 2 hours each). Paraffin blocks were sectioned at a thickness of 3 µm (five sections per block) using a microtome. Sections were mounted onto Silane-Prep slides (S4651-72EA, Sigma-Aldrich, USA), deparaffinized in xylene, rehydrated through a descending ethanol series, and rinsed in distilled water.

Histological and Immunohistochemical Analysis

Three sections from each tooth at each developmental stage (Ri, R1/2 and Rc) were selected for analysis.

Hematoxylin and Eosin (H&E) Staining

One section per sample was stained using a Hematoxylin and Eosin Staining Kit (ab245880, Abcam, UK) according to the manufacturer's instructions to provide an overview of the tissue morphology.

Immunohistochemistry (IHC)

wo sections per sample were used for IHC analysis to evaluate the expression of Neurofilament heavy (NFH) and Nestin. The following primary antibodies were used: rabbit polyclonal anti-Neurofilament heavy (NFH) (, Abcam, ab8135, lot#GR3183142-7, RRID:AB_306298) at a 1:5000 dilution, and mouse monoclonal anti-Nestin (Abcam, ab18102, lot #GR147064-44RRID:AB_444246) at a 1:100 dilution. Phosphate buffered saline (PBS, Abcam, ab286856) pH 7.4, was used in place of the primary antibody for negative controls.

IHC was performed manually in a humidity chamber. Antigen retrieval was achieved using Proteinase K Ready to Use (S302030-2, Dako, USA) for 3-6 minutes at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (216763, Sigma-Aldrich) in distilled water for 10 minutes.

Nonspecific binding was blocked with 5% normal goat serum (ab7481,Abcam) in PBS. Sections were incubated overnight at 4°C with the primary antibodies. Following washes with TBS-T wash buffer (20x TBS-T with Tween 20, ab64204, Abcam), sections were incubated for 30 minutes at room temperature with the appropriate horsera-dish peroxidase (HRP)-conjugated secon-dary antibodies (SignalStain Boost detection reagent (HRP, Rabbit) RTU, Cell Signaling, USA; or SignalStain Boost Detection Reagent (HRP, Mouse) RTU, Cell Signaling).

After further washes with TBS-T, the immunoreactivity was visualized using the SignalStain DAB Substrate kit (#8059, Cell Signaling) according to the manufacturer's instructions. Sections were then washed, lightly counterstained with Mayer's hematoxylin (S330930-2, Agilent Dako, USA), dehydrated, cleared in xylene, and coverslipped using SignalStain Mounting medium (Cell Signaling, USA).

Image Acquisition and Qualitative Analysis

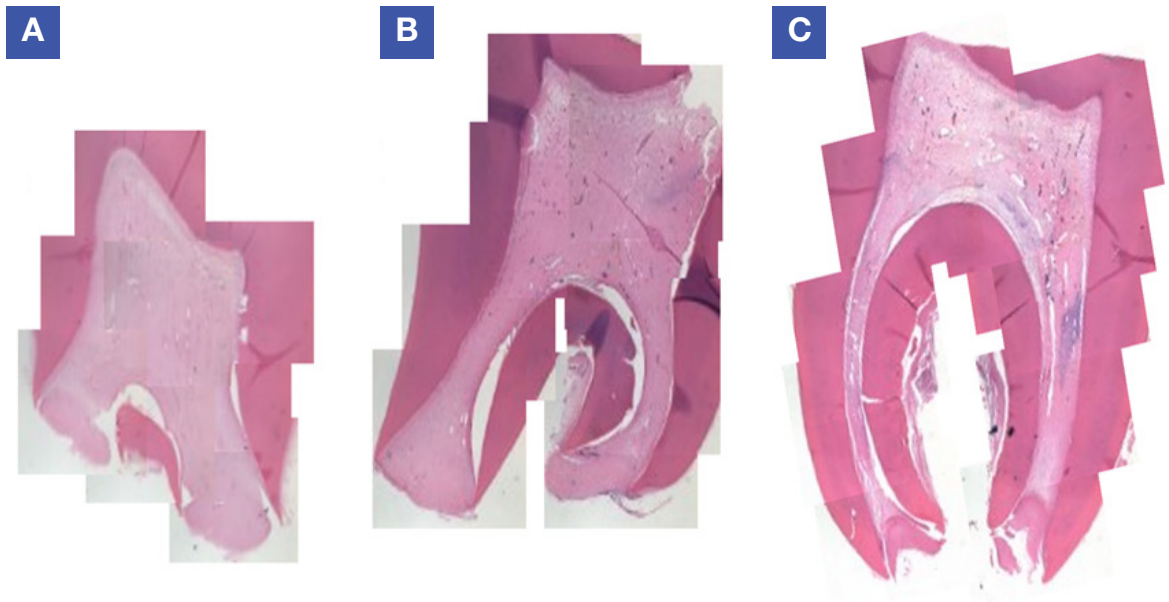
Photomicrographs of H&E-stained sections and IHC-labeled tissue sections from each developmental stage (Ri, R1/2 and Rc) were captured using a light microscope (Nikon, Eclipse Ti-5, Japan) equipped with an Infinity 3-6UR camera (Lumenera, Canada) at 10x, 20x, 40x, and 100x magnifications. For IHC, the spatial distribution of NFH and Nestin immunoexpression was qualitatively analyzed across the dental pulp tissue at each stage of root development.

RESULTS

Hematoxylin and eosin staining showing development of the root formation in each stage can be observed in Figure 1.
Immunohistochemical analysis of Neurofilament heavy (NFH) and Nestin revealed distinct expression patterns within the dental pulp tissue across all developmental stages (Ri, R1/2, and Rc). Control sections, as expected, exhibited no specific immunoreactivity for either antibody.

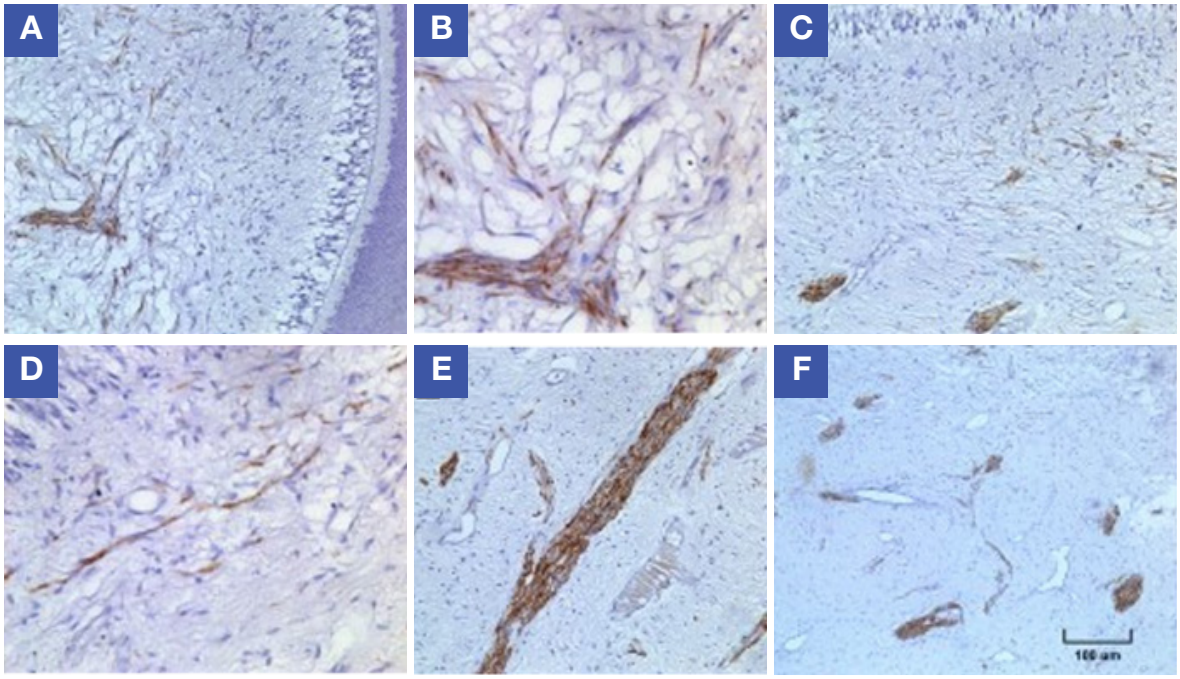
Neurofilament Heavy (NFH) Expression: NFH immunoreactivity was observed primarily in the core of the dental pulp, where it appeared as bundles of nerve fibers (Figure 2E, Figure 3A, Figure 3B, Figure 3C and Figure 3D, Figure 4B, Figure 4C and Figure 4D) and nerve sprouts (Figure 2F, Figure 3C and Figure 3D). In the peripheral regions of the pulp, NFH staining indicated the subdivision of axons into smaller branches, particularly between the pulp core and the cell-rich zone

Figure 1. Hematoxylin and eosin staining of developmental stage



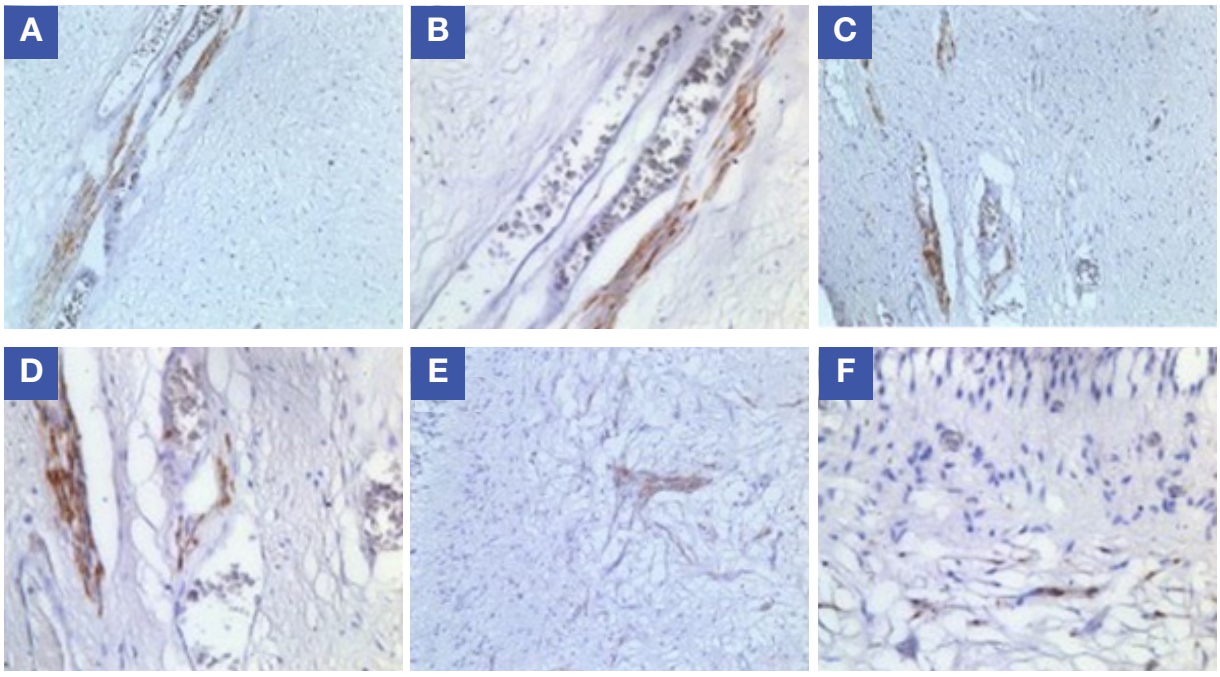
A. Ri specimen. **B.** R ½ specimen. **C.** Rc specimen. Stitched images from H&E sections to show development of the root formation in each stage.

Figure 2. Immunohistochemical assay for neurofilament heavy chain (NFH) on developmental **stage Ri**



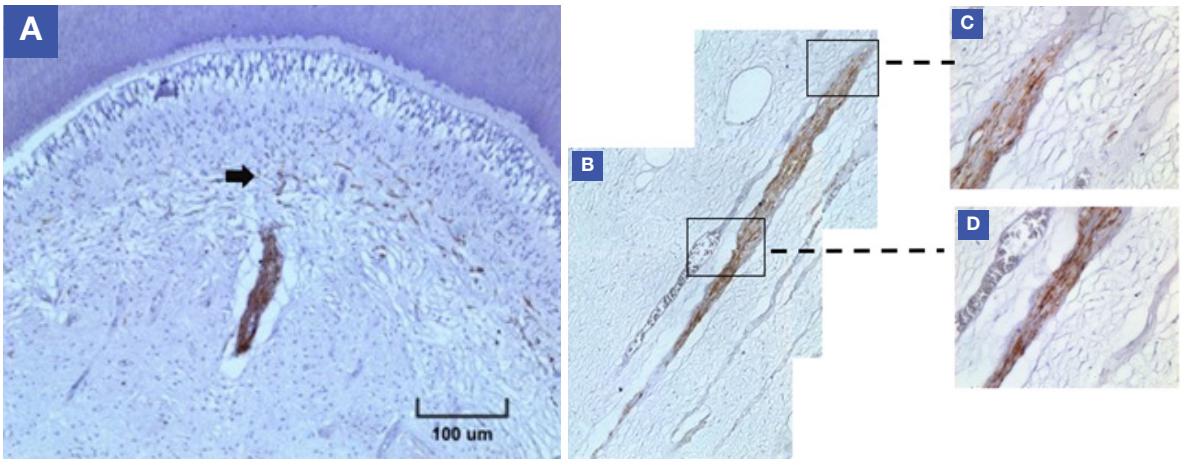
A AND B. Dental pulp in the peripheral areas, showing subdivision of the axons into smaller branches below the cell-rich zone. **C, D, E AND F.** Core of the dental pulp, showing bundles of nerve fibers, sprouts, axons and collateral branches. **Scale bar = 100 µm (A,C,E,F). Scale bar = 50 µm (B,D).**

Figure 3. Immunohistochemical assay for neurofilament heavy chain (NFH) on developmental **stage R ½**



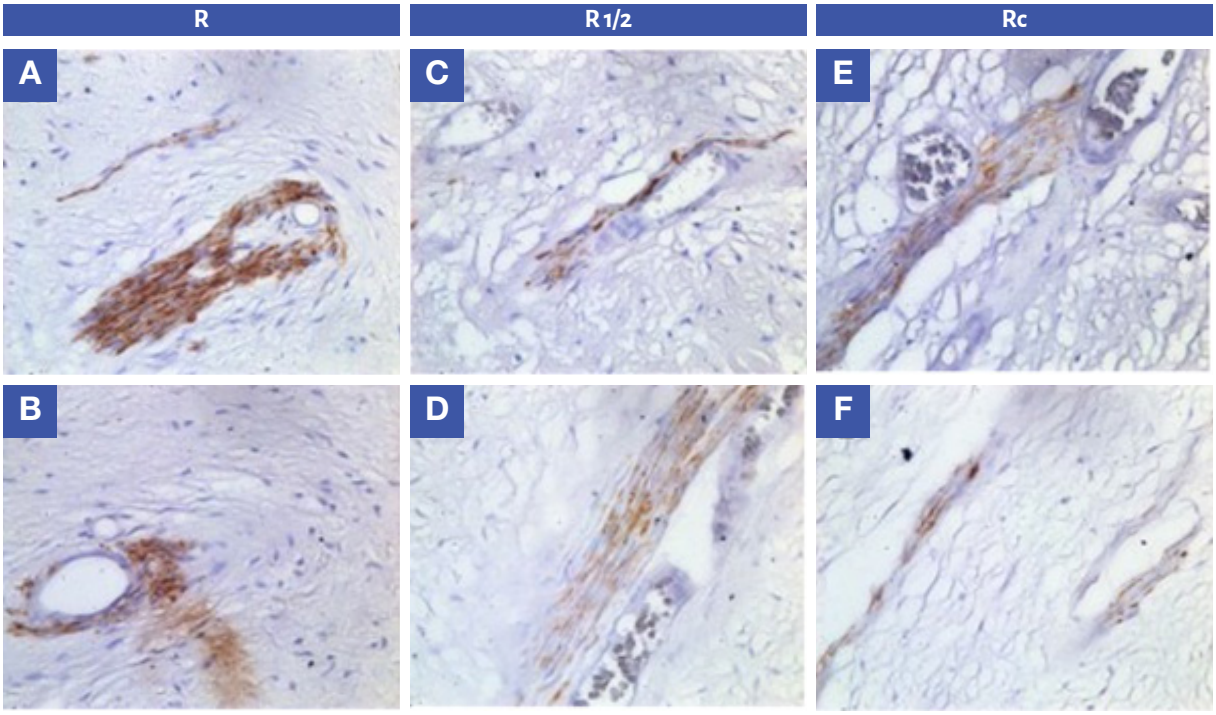
A, B, C AND D. Core of the dental pulp, shown axons together with vascular components. **E AND F.** Dental pulp in the peripheral zones shows sprouts and collateral branches in the tissue.
Scale bar = 100 µm (A,C,E). Scale bar = 50 µm (B,D,F).

Figure 4 . Immunohistochemical assay for neurofilament heavy chain (NFH) on developmental **stage R ½**



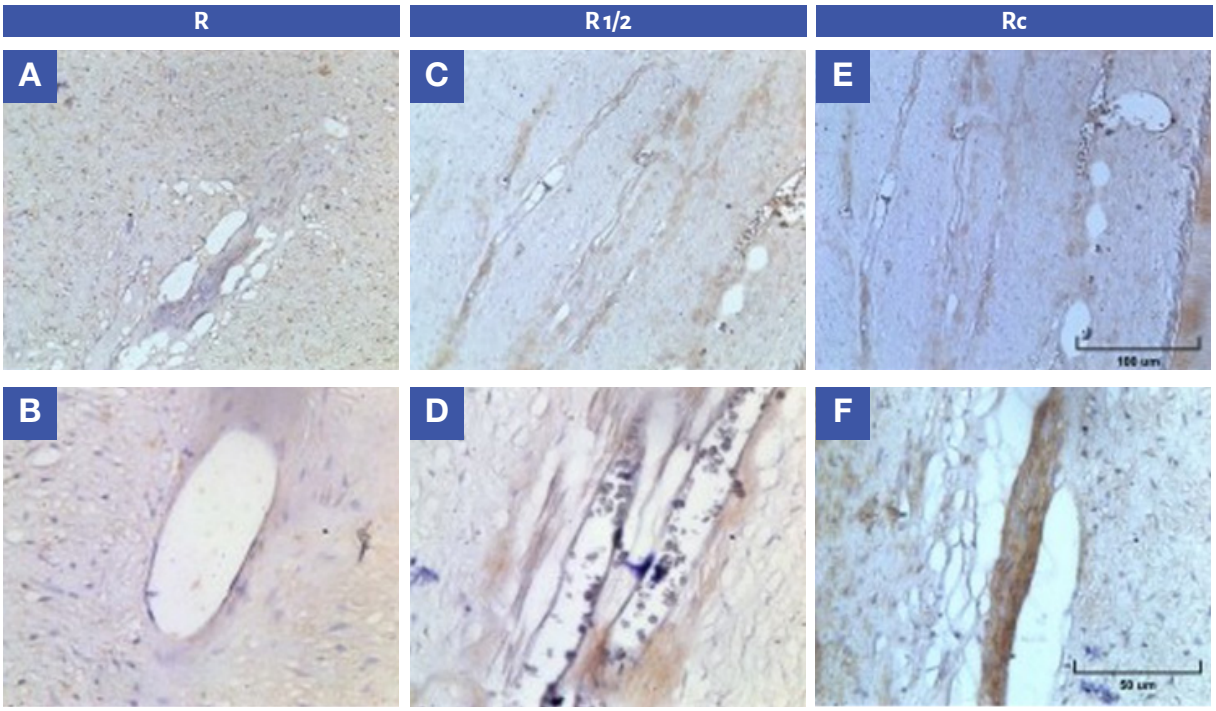
A. Dental pulp in the peripheral zones shown NFH staining below the cell rich zone, corresponding to the Rasckow's plexus. **B, C AND D.** Center of dental pulp, shown nerve bundles and mature axons in the tissue.
Scale bar = 100 µm (A).

Figure 5. Immunohistochemical assay for neurofilament heavy chain (NFH) on developmental stages. NFH observed together with the vascular structures along the pulp tissue in all the development stages



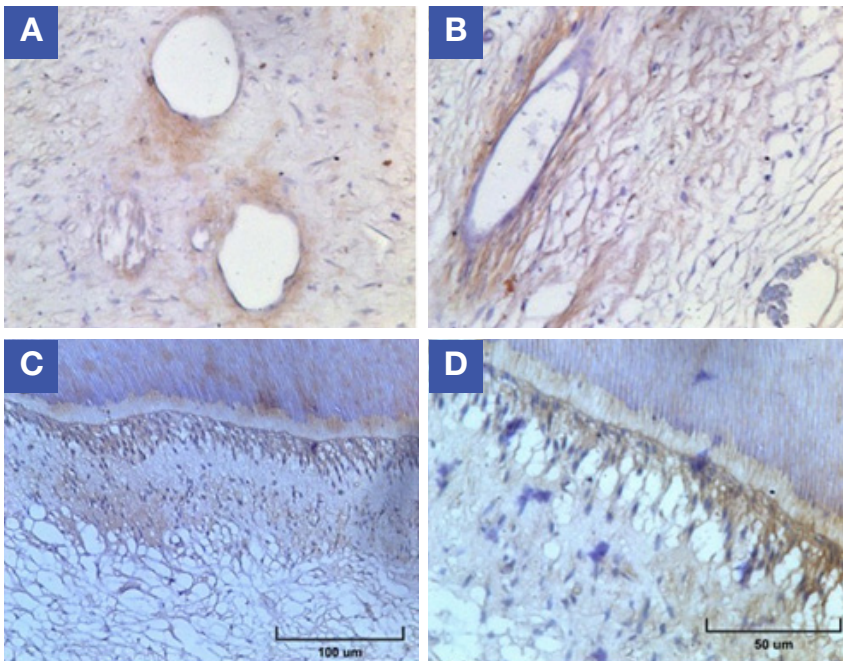
Scale bar = 50 µm (B,D,F).

Figure 6. Immunohistochemical assay for nestin on developmental stages. Nestin observed together with the vascular structures along the pulp tissue in all the development stages



Scale bar = 50 µm (B,D,F).

Figure 7. Immunohistochemical assay for nestin



A and B. Dental pulp in the core shown immunoreaction pattern for nestin together with vascular components. **C.** Peripheral dental pulp shows nestin in differentiated odontoblasts (OBs) in the OB layer, in the SOB layer and was uniformly stronger in OB cell processes in the predentin. **Scale bar = 100 µm (A).**

(Figure 2A, Figure 2B, Figure 2C and Figure 2D, Figure 3E, Figure 3F and Figure 4). Notably, NFH immunostaining was also consistently observed in close association with arteries and capillaries across all developmental stages (Figure 5).

Nestin Expression

Nestin immunoreactivity was detected alongside vascular structures throughout the pulp tissue in all developmental stages (Figure 6, Figure 7A and Figure 7B). In the peripheral area, strong Nestin expression was observed within the odontoblastic layer (differentiated odontoblasts), the subodontoblastic layer, and in association with odontoblast cell processes within the predentin (Figure 7C and Figure 7D).

DISCUSSION

Histological visualization of root formation stages, combined with the immunostaining of neural markers, is crucial for elucidating potential pathways for dental tissue regeneration. Dental pulp innervation, essential for pulp homeostasis, vascular and immunological regulation, repair, and regeneration, provides tissue stability during both natural development and regenerative processes.[5,6]

This study aimed to characterize the morphological aspects of dental pulp innervation through the expression patterns of NFH and Nestin. Understanding the complex innervation morphology is fundamental to unraveling the mechanisms of dental sensory perception and pain signaling. The rich nerve network within the dental pulp plays a critical role in transmitting mechanical, chemical, and thermal stimuli.[2,4,12]

NFH immunoreactivity was observed between the pulp core and the cell-rich zone (Figure 4A), suggesting the presence of nerves forming the Raschkow's plexus, consistent with descriptions by Golberg *et al.*,[12] Intermediate filaments as NFH, is an specific marker for myelinated axons, our findings are consistent with the reported progressive loss of myelin sheaths as axons approach the coronal pulp, culminating in free nerve endings near odontoblasts, and occasionally extending into dentin.[12,13] The observed NFH staining pattern also aligns with the reported termination of many axons below the odontoblast layer.

The majority of nerve axons that innervate teeth penetrate through the apex, via blood vessels.[4,12] The close association of NFH and Nestin expression with arterioles and venules in the central pulp, as observed in our study, supports previous findings that nerve fibers regulate blood flow and inflammation.[2,4,12] These intra-dental sensory axons may contribute to the maintenance and repair of the pulpo-dental complex by inducing vasodilation and inhibiting sympathetic vasoconstriction in response to pain. The observed sprouts, nerve fiber bundles, and collateral branches along the pulp tissue suggest ongoing development of the innervation system, correlating with pulp tissue maturation and root formation, as we previously reported.[6,8]

Nestin, an intermediate filament predominantly expressed during development and associated with stem cell functions (self-renewal/proliferation, differentiation and migration).[9] Expressed in multipotent neuroectodermal precursors and suppressed during subsequent development. Therefore, it can be used as an early marker for the differentiation of precursor cells.[14]

In our study, it was found predominantly in the odontoblastic lineage cells (odontoblastic layer, subodontoblastic layer, and odontoblast cell processes in predentin). This suggests the presence of progenitor cells in the subodontoblastic layer, crucial for odontoblast replacement, pulp homeostasis and response.[6] The observed Nestin expression in odontoblast cell processes aligns with reports of its specificity for this cell lineage.[12,15]

Nestin expression alongside vascular structures in the pulp core across all developmental stages may indicate endothelial cell expression of Nestin[9] and its involvement in dental pulp healing after injury.[10,16,17] This suggests a potential role in dental pulp development, although the underlying mechanisms remain unclear.

A comprehensive understanding of neuronal marker expression and localization in the dental pulp is essential for deciphering the neural components involved in pulp function and its response to stimuli. Successful tissue regeneration relies on the formation of a functional vascular network and innervation.[4,18] Achieving structural and functional similarity to natural dental pulp in regenerative processes[18,19] necessitates a thorough understanding of these biological features.

To further clarify the mechanistic contributions of NF-H and Nestin to human dental pulp development, it is important to consider their established roles in other neural and developmental contexts. NF-H, as a cytoskeletal protein, directly influences axonal growth and stability, which is essential for the formation of a functional neural network within the pulp.[20] Specifically, NF-H contributes to the radial growth of axons and the maintenance of their caliber, crucial for proper signal transduction. Nestin, on the other hand, acts as a marker and regulator of progenitor cell populations,[21,22] influencing their differentiation into odonto-blasts and potentially contributing to the regenerative capacity of the pulp.[21]

In the context of dental pulp development, Nestin's expression in odontoblast lineage cells suggests its role in maintaining a progenitor pool for dentin formation and repair.[6,12,15] The co-localization of these markers suggests a complex interplay, where neural development, guided by NF-H, and progenitor cell activity, regulated by Nestin, are tightly coordinated to ensure proper pulp formation and function.

The findings of this initial screening provide valuable preliminary insights that warrant further investigation using quantitative methodologies and larger sample sizes in subsequent studies. Future studies employing advanced molecular techniques are needed to fully elucidate the signaling pathways and interactions involved. From a treatment perspective, these findings suggest that understanding the precise roles of NF-H and Nestin in dental pulp development and regeneration could lead to novel therapeutic strategies.
Future research should explore these potential clinical applications, ultimately aiming to develop more effective regenerative therapies for dental pulp.

CONFLICT OF INTERESTS

The authors have no conflicts of interest.

ETHICS APPROVAL

Research protocol was approved by the Ethics Committee of the University of Costa Rica, San José, San Pedro, Costa Rica (Protocol Number: VR-467-2018). All procedures adhered to the ethical principles outlined in the Declaration of Helsinki (1975).

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AUTHORS' CONTRIBUTIONS

Cristina Retana-Lobo: Conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization.

Tatiana Ramirez-Mora: Validation, formal analysis, writing—review and editing, and final approval of the version to be published.

Jessie Reyes-Carmona: Conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing-review and editing, visualization, supervision, project administration, funding acquisition and final approval of the version to be published.

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