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RESEARCH ARTICLE

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VASCULAR AND NERVOUS CHANGES IN DENTAL PULP OF HUMAN TEMPORARY TEETH ASSOCIATED WITH THE PHYSIOLOGICAL ROOT RESORPTION PROCESS

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ABSTRACT

Objective: To determine vascular and nervous changes during the progression of physiological root resorption, in three stages.

Materials and methods: 21 healthy temporary canines with indication of extraction, conducted on the Faculty of Dentistry of the University of Valparaiso were analyzed. Samples were processed for indirect immunofluorescence using Alexa Fluor 488 goat anti-mouse fluorochromes for Schwann cells (CS); Alexa fluor 555 goat anti-rabbit for endothelium. By confocal microscopy, a subodontoblastic portion, greater than the amelocemental junction, and an apical portion related to the root resorption area were analyzed. They were classified in initial, middle, and advanced stages. To process images, Ez-C1 3.90 and ImageJ programs were employed, and through visual analysis, researchers described pulpal changes.

Results: As the tooth was resorbed, nervous tissue degraded and disorganized with angiogenesis around it during middle stage. The same was observed in the coronal section, but with more predominance in the apical section. As physiological root resorption happened, the vascularity of temporary teeth increased.

Conclusion: As physiological root resorption ensues, simultaneously occurs degradation and disorganization of nervous tissue, along with angiogenesis.

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KEYWORDS:

Tooth, deciduous; root resorption; wallerian degeneration; blood vessels; fluorescent antibody technique.

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INTRODUCTION

The transition from temporary to permanent teeth is a unique and dynamic process in which the development and eruption of permanent teeth coordinate with physiological root resorption (PRR) of temporary teeth[1], this complex phenomenon is yet to be fully explained, and it is still of great clinical and biological interest[2].

Many pulp histological changes occur in temporary teeth because of this phenomenon. Changes associated with innervation and inflammatory infiltrate have been previously described[2,3], but, regarding pulp vasculature, both blood and lymphatic, there is not enough information that allows creating an overview of what happens during this process.

The purpose of this study is to determine vascular changes through comparative immunohistochemical analysis and confocal microscopy to achieve a further understanding of vascular-nervous changes during PRR on human temporary teeth, so that is possible to interpret the resorptive phenomenon and extrapolate its factors to pathological scenarios that affect the primary dentition.

MATERIAL AND METHODS

A cross-sectional, descriptive observational study was conducted on vascular-nervous changes in the dental pulp of temporary canines undergoing PRR.

Twenty-one asymptomatic temporary canine teeth without caries with different degrees of PRR were collected between August 2017 and January 2018. Prior donation of the teeth, informed consent was asked to the adult caretaker and the assent from the child was obtained, all canines had indications for extraction. Inclusion criteria were systemically healthy patients with temporary or mixed dentition, normal chronology, temporary healthy teeth with an indication of extraction endorsed by an orthodontist, with its crown intact and superficial lesions-free. Regarding the exclusion criteria applied, these were: teeth undergoing pulp necrosis, irreversible pulpitis, residual root, temporary teeth with a history of dental trauma, or avulsion, temporary teeth suffering from active/arrested caries, ankylosed canine or with an altered chronology of eruption.

All donors were patients of the Faculty of Dentistry of the University of Valparaíso, attended in the General Children's Dentistry clinics (I and II), aged 5 to 12 years, inhabitants of the Valparaíso Region, Chile.

The samples were classified into 3 groups, according to their PRR: initial stage, middle stage, advanced stage, based on stages described by Moorees in 1963 (4). The groups were obtained by the difference between maximum root length (13.25 mm) and minimum (1.5 mm) of the teeth, and dividing it into 3 (13.25 - 1.5 / 3), with this, it was obtained a 4mm approx. variability in each stage. For the sample selection, the PRR had to be restricted mainly from apical, with at least 1mm of root remnant. In contrast, all samples that presented poor fixation posterior processing, oblique or horizontal PRR, or teeth showing loss of pulp tissue, were excluded.

For the gathering of the samples, the investigator present during the exodontia took the tooth for examination, in search of pulp exposure. In those teeth who didn't present exposure, a transversal cut was performed mid-crown using a high-speed diamond bur. Then it was deposited with the pressure tweezers in a conical tube containing 20ml of 4% paraformaldehyde plus 0.5% picric acid for its fixation during 6 hrs., The tubes were identified employing an alphanumeric code (i.e.: A1, A2, ...). Once completed the fixation time, the samples were demineralized in a 4.13% EDTA solution, which was changed once a week, repeating this process for 3 months, until it was possible to start the necessary processes for cutting tests, immunohistochemistry and confocal microscopy.

Demineralized samples were washed with a phosphate-saline buffer solution (PBS) and cryoprotected in PBS solution with 15% sucrose for 2 hours, then this step was repeated in the same 30% solution for 24 hours.

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Samples soaked in a cryopreservative solution were mounted in tissue freezing medium (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA, USA) and frozen at -25C°. 25um cuts were performed in a cryostat (Leica CM-1900) operated at -25 ° C and then mounted on slides previously positively charged with poly-lysine; these sections were rehydrated in PBS and incubated for 1 hour in a blocking solution composed of 1% bovine serum albumin (BSA), 1% horse serum, and 0.3% Triton X-100. Then the primary antibodies were added, which were diluted in a blocking solution and left to act for 12 hours at a temperature of 4 ° C. Samples went through 3 PBS washes of 10 minutes each. Then the secondary antibodies were added, also diluted in a blocking solution (1: 500) they were left to act for 1 hour at room temperature; later, it was washed again with PBS to remove excesses.

Subsequently, the nuclei were marked with DAPI (0.25µg / ml) for 10 minutes, finally, the samples were washed and covered with a mounting medium (Dako Industries, Carpenteria, CA, USA). For their maintenance, the samples were kept in plastic boxes at 4 ° C. Processed samples were analyzed with a Nikon C1 Plus confocal microscope.

The investigators used three lasers of different emission wavelengths (405, 488, and 555nm). The fluorochromes associated with the secondary antibodies were Alexa Fluor 488 goat anti-mouse (in green), Alexa Fluor 555 goat anti-rabbit (in red), and DAPI (405nm).

Digital records were processed employing Ez-C1 3,90 (Nikon Corporation) and Image J (NIH, Bethesda, MD, USA) programs for maximum intensity projections. For means of contrast and brilliance, Adobe Photoshop CS4 (Adobe Systems, Mountain View, CA, USA) was selected. For a standardized analyze, it was decided to use two base zones of 400 x 400 µm, zone 1 corresponded to the coronal pulp, located in the sector of the subodontoblastic portion superior to the amelo-cemental limit; meanwhile, zone 2 corresponded to an area of the same size in the most apical portion of the tooth, related to the root resorption zone itself.

Using the ImageJ processing program for multidirectional scientific images, the CS and endothelial images were quantified in an analog way by the researchers using confocal images, which were obtained from longitudinal sections of the dental pulp of twenty-one teeth.

In these immunoreactive profiles, it was fixed an expression threshold in all images obtained; At threshold fifty, the background elimination of the photographs of this investigation was achieved. The colors seen in the images correspond to red endothelial cells and green nerve cells.

The variables of this study were the type of tooth (temporary teeth, teeth that erupt between approximately 8 to 30 months of age, which after undergoing the physiological exfoliation process, were to be replaced by permanent teeth), the degree of radicular resorption (identifying the stage of PRR of temporary teeth, established by 3 stages (initial, middle, advanced)), vascular markers (identified by antibodies CD31, CD34, vWF, CD105), and markers of nerve components (identifiable due to the immunoreactivity of the CS against the S100 marker).

This study was approved by the Institutional Bioethics Committee for Research in Human Beings of the University of Valparaíso and complies with all the principles and updates of the Declaration of Helsinki.

RESULTS

After the inclusion and exclusion criteria were applied, 21 eligible samples were obtained, confirmed for processing.

Subsequent to the qualitative analysis, important changes were evident during the progression of the PRR when comparing the three stages. Vascular-nervous tissue increased significantly in this zone, especially when going from medium PRR to an advanced PRR. (Figure 1, Table I)

By schematizing the findings, considering the amount and density of tissue in each sample and

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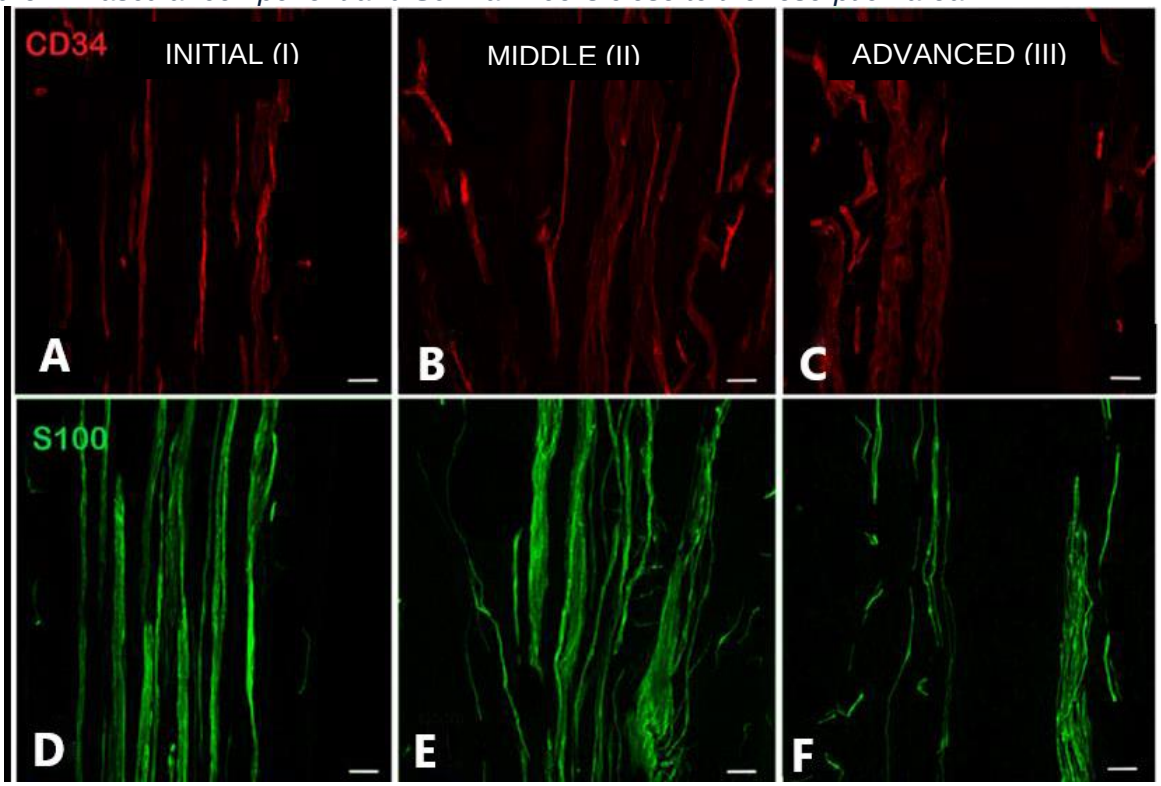
Figure 1: Vascular component and Schwann cells close to the resorption area



Vascular component (CD34, red) and Schwann cells (S100, green). Close to the resorption area, the fascicular degeneration process of the nervous component is observed as the resorption process progresses. In the initial PRR, a linear and neat fascicular arrangement of the axons is observed (D); In the middle PRR, an increase of the vascular caliber is observed (B) this persists in the advanced PRR (C) Furthermore, in this stage a fragmented fascicular disposition is observed (F). The increased vascular caliber is distributed around the areas of axonal degeneration (E, F). Scale bars: 50 µm.

Table I: Schematization of the samples close to the resorption area

Radicular Pulp	Initial PRR	Middle PRR	Advanced PRR
Schwann Cells (S100)	+ +	+ + +	+
Vascular Components (CD34)	+	+ + +	+ +

The schematization of the samples considering the quantity and density of tissue according to their PRR stages in the area close to the reabsorption. The more (+), the more predominance of the tissue.

classifying it based on its stage of PRR; the nervous tissue becomes more prevalent in the middle stage; this is due to its fascicular disorganization and because of its degradation it decreases towards the advanced stage. As for the vascular component, it increases on the middle stage, the same as SC, and in the advanced stage decreases its distribution the same as nervous tissue. In the areas with no nervous tissue, there is also no predominance of vascular tissue, establishing a correlation (Table I).

The same vascular-nervous changes observed in the area close to the resorption are observed in the coronal pulp, above the amelo-cemental junction, but more attenuated. (Figure 2 and 3)

By schematizing the findings, considering the quantity and tissue density, it was observed that nervous tissue increases in the middle stage, due to disorganization of the fibers, also it was observed their degradation, which in the advanced stage is almost complete. Vasodilation and vascular neoformation characterized the

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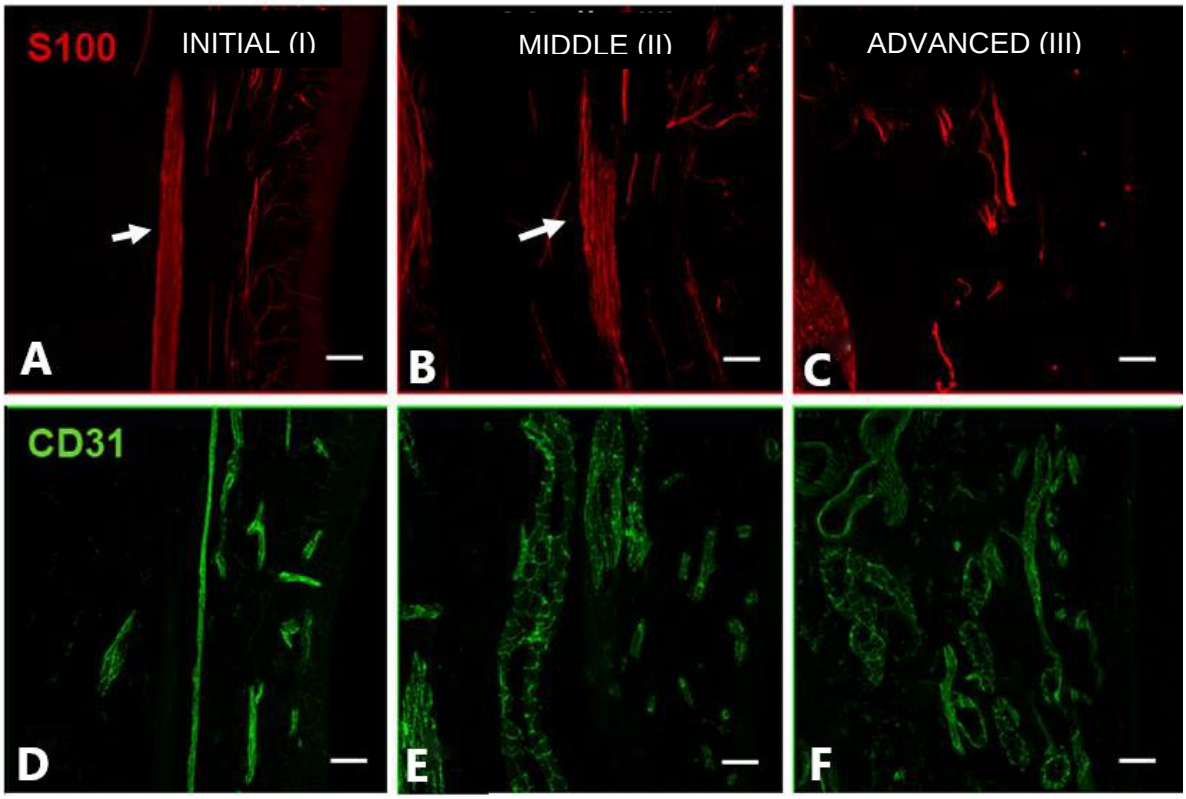
Figure 2: Vascular components and Schwann cells of coronal pulp above the amelo-cemental junction during PRR



Characterization of vascular components (CD31, green) and Schwann cells (S100, red) of coronal pulp above the amelo-cemental junction during PRR. In the initial stage, an integrated and conserved axonal band is observed (A, arrow); In the middle stage, myelinated axons bands are disintegrated (B, arrow) as the process progresses. Vasodilation is observed, with foci of angiogenesis (E); in advanced PRR, the axonal band degenerates losing its structure (C) also it is observed marked vasodilation, changes in the vascular pattern with distribution around areas of axonal damage (F). Less presence of nervous tissue. Scale bars 50 µm.

Table II: Schematization of the samples considering the quantity and density of tissue in the coronal pulp, over the amelocemental junction, according to their PRR stages.

Coronal P.	Initial PRR	Middle PRR	Advanced PRR
Schwann Cells (S100)	++	+ + +	+
Vascular Components (CD34)	+	+ + +	+ + +

The more (+), the more predominance of the tissue.

passage to the middle stage of PRR, together with the beginning of the degradation of the myelinated axon bundles, increasing its preponderance, this vasodilation and growing angiogenic zones develop fully in the advanced stage. In advanced PRR, angiogenic foci are observed in the reabsorption zone, and axonal degradation in the center and periphery (Table II).

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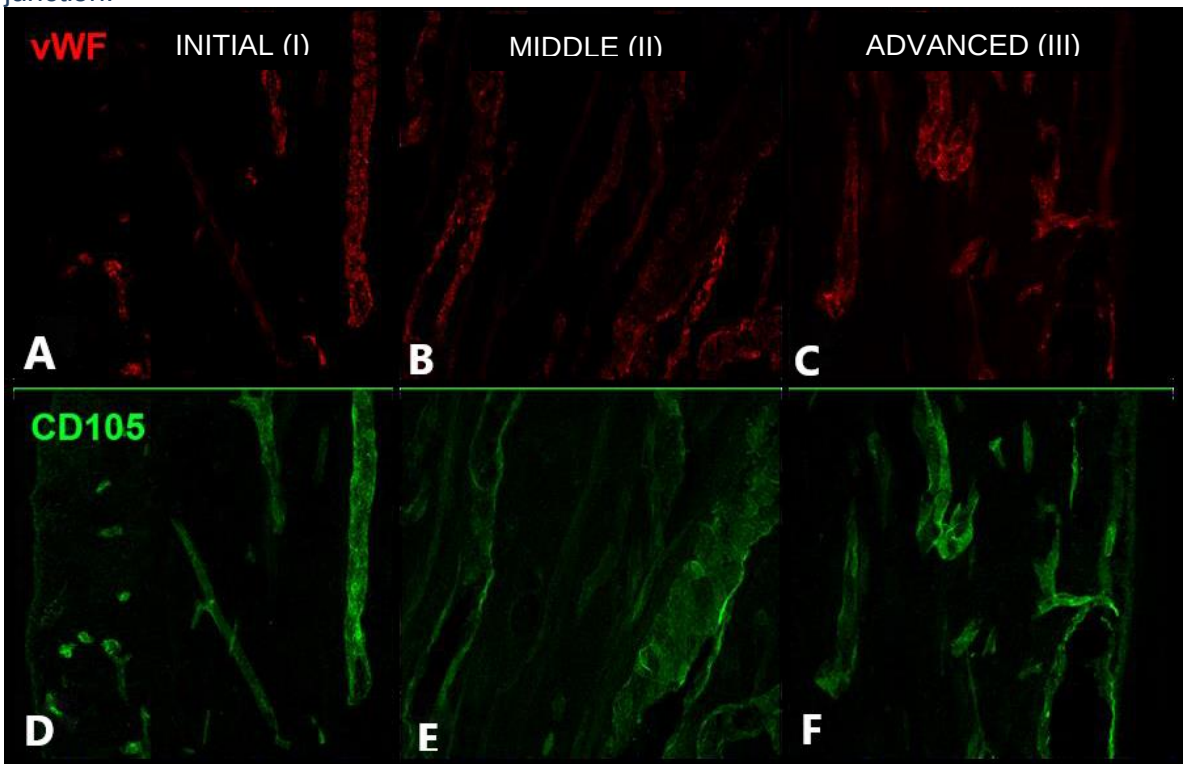
Figure 3: Double labeling for endothelial cells of the coronal pulp on the amelocemental junction.



Double labeling for endothelial cells (vWF, red; CD105, green) of the coronal pulp on the amelocemental junction. In the middle stage of PRR, the greatest increase in vascular diameter (B, E) is shown, accompanied by the proliferation of endothelial cells, which is also present in the advanced stage (C, F).

DISCUSSION

The main finding of this study was the variation in marking at the level of endothelial and nerve cells.

As the PRR progresses, the behavior of the SC changes in its distribution and increases in its concentration in the initial and middle stages of the PRR process, the progress of this phenomenon is associated with an asymptomatic chronic inflammatory process, as described by Angelova et al.[17]. This process comprehends an axonal degeneration and progressive denervation of the dental pulp in temporary teeth, which reflects a loss of the myelin sheath, leading to a differentiation of SC, which allows activation of the phenotypic plasticity of these cells, towards a repairing phenotype (RSC)[8] RSC promotes the remodeling of the degenerated axons of the dental pulp, through an autophagic pathway for the removal of myelin debris from axonal degeneration[9], this phenomenon is observed in the initial and middle stages during PRR, this matches with what was witnessed in this study, where a higher SC marking was obtained in the middle stage, being comparable to the research by Suzuki et. Al.[3], considering a similar methodology was employed with statistically significant results achieved. This higher concentration of SC (both coronal and apical of the dental pulp), is influenced by a greater degradation of the myelin sheath, therefore, a greater manifestation of RSC in an attempt to remodel the nerve fibers of the dental pulp.

In advanced stages, there is a larger expression of RSC for the correct remodeling of damaged axons, centering this phenomenon in the coronal area of the pulp according to what was stated by Couve et al.[10], is because of this that temporary teeth undergoing PRR may present pain when presented to some type of noxa or stimulus, for this, it is recommended the use of local

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anesthetic for dental procedures on temporary teeth experiencing an advanced PRR[11].

As for the endothelial cell marking, what stands out is that there is a similarity with the marking of degenerative areas of nerve cells, this is because along with nerve degeneration occurs angiogenesis, which leads to qualitative differences in the distribution and morphology of blood vessels, concentrating these changes in the coronal area, according to Monteiro Et. Al[11], that study differs in methodology and does not produce statistically significant results, compromising any extrapolation of their results, but in terms of biological plausibility, greater angiogenesis reflects a greater metabolic requirement during the PRR process, since it is required a greater blood flow for the subsistence of this phenomenon and, subsequently, the eventual dispose of metabolic residues[12].
Among the limitations of this study was the limited number of samples and the non-randomness of this, despite of that, the processing of the samples was carried out by the ImageJ software, which has been used by numerous investigations at an international level because it allows an objective and finished processing of the images obtained, in addition, the analysis of the samples was jointly carried out with the aid of an expert observer on the subject, who is credited with vast years of experience and multiple investigations validated in the scientific community.

Another limitation of this study was the PRR categorization employed, that, even though it has been previously used in past studies[4], was modified and is deeply influenced by the collected samples of the study, because of that this classification was drastic and broad (⅓, ⅓ - ⅔, ⅔ - > ⅔), this leads to possible correlations between the exact percentage of root resorption and the pulp state being masked, which causes a selection bias. For posterior investigations, we suggest the implementation of an internationally validated guide or one validated in another study.

Erroneously, it is considered that temporary teeth undergoing PRR are less sensitive to pain, thus, performing dental treatments avoiding the use of local anesthesia, however, current scientific evidence attests that these teeth preserve their regeneration and defensive capabilities well into PRR, this must be taken into consideration in the management of pain in pediatric dentistry, to preserve the primary dentition using conservative methods[3], moreover, nowadays temporary teeth are one of the main sources of stem cells, which are forebears of tissues with therapeutic expectations in many diseases in the future[4,6].

This study was focused on temporary teeth with different degrees of PRR, for this reason, it would be necessary to execute further investigations on how teeth suffering from caries, trauma or lesions, besides undergoing PRR ,behave when subjecting them to clinical procedures, to compare pulp resistance (which directly influences the prognosis of the tooth).

CONCLUSION

In conclusion, as PRR ensues, simultaneously occurs degradation and disorganization of nervous tissue, along with angiogenesis based on the results obtained, this implies that dental pulp retains its vitality during PRR unto more advanced stages. With this knowledge, we can employ new guidelines in the decision of the treatment plan in teeth undergoing PRR.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with respect to this article.

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IN MEMORIAM EDUARDO COUVE

"Trabajar con Dr. Eduardo Couve fue un privilegio. Su capacidad innata para contagiar la pasión por la ciencia y el aprendizaje en cualquier categoría es una habilidad que nunca antes vimos en otro docente. Agradecemos profundamente su dedicación, su amor por lo que hacemos y sobretodo su actitud cercana, que propició un ambiente académico casi familiar, que jamás olvidaremos. Su partida tan prematura es una real pérdida para la universidad, siempre será extrañado. QEPD querido Dr. Couve" (Diego Escudero, Macarena Orellana, Daniela Encalada).