Vascularity and VEGF/VEGFR2 Signaling in the Dentine–Pulp Complex of Immature and Mature Permanent Teeth

Lara T FRIEDLANDER, Dawn COATES, Gregory SEYMOUR, Mary CULLINAN, Alison M RICH

ABSTRACT

Objective: To examine the microvessel density (MVD) and spatial distribution of endothelial cells and angiogenic activity in immature and mature permanent teeth using immunohistochemistry.

Methods: Healthy third molars with immature and mature root development were formalin-fixed, decalcified in 10% ethylenediaminetetraacetic acid, and processed for routine immunohistochemistry with endothelial cell markers anti-CD34 and anti-CD146 and angiogenic markers anti-vascular endothelial growth factor (VEGF) and anti-VEGF receptor-2 (VEGFR2). Staining was visualized with diaminobenzidine and examined using light microscopy. The distribution of markers was analyzed qualitatively and quantitatively in the coronal, middle, and apical regions of the dentine–pulp complex.

Results: There were spatial differences in protein expression for immature and mature teeth. The pulps of immature teeth were more vascular, had a greater number of CD34+ and CD146+ cells, and a significantly higher MVD in the coronal region than those of mature teeth (P=0.03). The apical papilla contained few blood vessels. VEGF/VEGFR2 activity was significantly greater for immature teeth (P=0.001). VEGF was expressed throughout the pulp–dentine complex, but there was significantly more growth factor coronally (immature P=0.04 and mature P=0.02). VEGFR2 was expressed less than VEGF but was seen on the endothelial cells and single cells unrelated to a vessel lumen.

Conclusion: The spatial distribution of vascular and angiogenic (VEGF/VEGFR2) markers indicates the potential for altered healing responses in the pulps of immature and mature teeth. Immature teeth have a greater MVD and VEGF/VEGFR2 expression than mature teeth, and the increased expression of these markers in the coronal region of both tooth types is important for pulp healing.

Keywords: Angiogenesis, histology, immature teeth, mature teeth, vascularity, VEGF, VEGFR2

INTRODUCTION

The loose connective tissue of the dental pulp is embryologically and histologically related to the dentine surrounding it, giving rise to the dentine–pulp complex. Furthermore, the pulp’s main link with the surrounding tissues is via the root apex, and the ability of the dentine–pulp complex to respond to injury by reactionary and reparative dentine deposition is well accepted. Healing may occur by regeneration, but reparative healing involving fibroblast-like cells is more likely and is dependent on growth factors and blood supply (1). Pulp healing is often unpredictable as, although the tissue is richly vascular, it is vulnerable owing to a lack of collateral blood supply (2).

Injury to immature permanent teeth from trauma or dental caries can result in favourable healing. Factors influencing outcomes are largely unclear, but Hertwig’s epithelial root sheath, progenitor cells, and a vascular supply are
important (3,4). These teeth have open apices that are adjacent to a blood supply, and most have a short distance from the crown to the root apex. Furthermore, the apical papilla tissue beneath the mature radicular pulp at the developing apex is unique to immature permanent teeth and has a recognized regenerative potential (5). It contains a rich source of stem cells and is thought to be the source of primary odontoblasts in root development (6). One report demonstrates healing and apexogenesis following vital pulp therapy for immature permanent teeth diagnosed with reversible and irreversible pulpitis, but this response can be variable (7). Conversely, in teeth with complete root development, the increased distance from a coronal pulp injury to the apical foramen, as well as the small size of the apical foramen in mature roots, limits the main vascular supply entering the pulp. Loss of pulp vitality is more frequent in teeth with fully developed root teeth, with pulp healing dependent on the ability of the circulation to recover (8).

Angiogenesis involves the growth of new vessels from the pre-existing capillaries and is fundamental to normal development and healing (9). The endothelial cells lining the pulp vasculature play a major role in the angiogenic process, and when injured, cytokine release stimulates the inflammatory response, resulting in the recruitment of progenitor cells to the site of injury to participate in the healing process (10). Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic cytokine secreted by many cell types and is a key regulator in physiological and pathological angiogenesis. VEGF acts on the vasculature by inducing the proliferation, differentiation, and migration of vascular endothelial cells. It has been identified in the dental pulp cells and the dentine matrix, where under pathological conditions, the growth factor can be released to stimulate angiogenesis (11–13).

Understanding of angiogenesis in the pulp–dentine complex is still in its infancy, and most studies have considered the effects of angiogenenic growth factors, but these responses depend on binding of growth factors to tyrosine receptors. Vascular epithelial growth factor receptor-2 (VEGFR2) has a dissociation constant (Kd) value of 75–125 pM, and as such, it is the highest affinity receptor for VEGF. This pro-angiogenic receptor is predominantly expressed on the surface of endothelial cells (14). Although VEGFR2 has been identified in the coronal pulp of primary and young permanent human teeth (15), there is limited knowledge about this receptor in the other regions of the pulp and apical papilla (16).

The endothelial cells have a primary role in angiogenic signaling between VEGF and VEGFR2. The aim of the present study was to examine the microvessel density (MVD) and spatial distribution of endothelial cells and the angiogenic activity in permanent teeth with immature and mature root development. Immunohistochemistry was performed using anti-CD34 and anti-CD146 antibodies to identify the endothelial cells and blood vessels and anti-VEGF and anti-VEGFR2 antibodies for the expression of these angiogenic markers in the dentine–pulp complex.

### MATERIALS AND METHODS

#### Tissue specimens

Twenty unerupted and impacted human third molar teeth were extracted for clinical reasons from patients aged 16–23 years. Samples were obtained from patients treated at the Faculty of Dentistry, University of Otago, Dunedin, New Zealand. All procedures were in compliance with the Lower South Regional Ethics Committee, New Zealand (Project no.: LRS/08/06/028). Samples with a history of local infection were excluded from the study. Ten immature permanent teeth had two-thirds root development with open root apices and attached apical papilla tissue, whereas the other ten teeth were mature and had fully developed root apices. After surgical removal, teeth were fixed in 10% neutral buffered formalin for 48 h prior to being decalcified in 10% ethylenediaminetetraacetic acid solution (pH 7.4). Specimens were routinely processed and paraffin embedded. Four micrometer longitudinal sections were cut and mounted on positively charged slides.

#### Immunohistochemistry methods

Monoclonal mouse primary antibodies anti-CD34 (3.1 µg/mL; Abcam, USA), anti-CD146 (20 µg/mL; Abcam), anti-VEGF (4 µg/mL; Santa Cruz Biotechnologies, USA) and polyclonal rabbit anti-VEGFR2 (8 µg/mL; Abcam) were used to examine the protein expression in the tissue samples. Antigen retrieval using a preheated 0.01 M citrate buffer (pH 6) at 85 °C for 10–15 min was used for anti-CD146, anti-VEGF, and anti-VEGFR2 in order to break the protein crosslinks formed during formalin fixation. No retrieval was required for anti-CD34. Endogenous peroxidase activity and non-specific binding were inhibited by immersion of the sections in 0.3% hydrogen peroxide in methanol for 10 min, followed by 20% rabbit serum/1% BSA/PBS (anti-CD34, anti-CD146, and anti-VEGF) or 20% goat serum/1% BSA/PBS (anti-VEGFR2). The primary antibodies were incubated overnight at 4 °C. For negative control sections, non-specific mouse IgG (Santa Cruz Biotechnologies) replaced monoclonal mouse primary antibodies, whereas non-specific rabbit IgG (Santa Cruz Biotechnologies) replaced anti-VEGFR2. These markers have been identified in pyogenic granuloma tissue that was used as a positive control tissue (17,18).

Thereafter, the sections were incubated with the secondary reagent EnVision™ HRP system (anti-mouse or anti-rabbit; Dako, Glostrup, Denmark) for 30 min at room temperature. Staining was visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA), and sections were counterstained with hematoxylin, and resin mounted (Entellan®; Electron Microscopy Sciences, Hatfield, USA).

#### Data analysis

Slides were evaluated using light microscopy at varying magnifications (up to 1000×), and the distribution of markers was analyzed in the coronal, middle, and apical regions. The coronal region was defined as the dentine–pulp tissue above the cementoenamel junction, whereas the apical region included 2–3 mm of apical root (dentine and mature pulp tissues). The apical region was defined as the apical cell rich zone (CRZ) of the mature pulp and the apical papilla in immature teeth. The middle region was between the coronal and the apical zones.
Quantitative analysis of markers was performed on the coronal and middle pulp regions and apical papilla of immature teeth and the coronal and middle pulp regions of mature teeth to enable comparisons within samples and between the two tooth types. The apical regions of immature and mature teeth were not directly comparable for quantitative analysis. Immunopositivity for anti-CD34 and anti-CD146 was used to determine the MVD and to identify the endothelial cells. Morphological structures that had a lumen and were lined by CD34+ endothelial cells were deemed to be blood vessels. The cells expressing VEGF+ and VEGFR2+ were quantitatively analyzed, whereas the expression in the dentine matrix was analyzed semi-quantitatively using a similar method used by Artese et al. (11) for the evaluation of staining intensity in representative fields. VEGF expression in the dentine matrix was graded as −, +, and ++ representing absent, low intensity, and high intensity staining, respectively (11). The coronal, middle, and apical regions of each section were examined. Images (area: 0.45 mm²) of three fields per region were evaluated from 20 sections of immature (n=10) and mature (n=10) teeth. Images were captured at 400x magnification using the SPOT™ Advanced Software, version 4.7 (SPOT™ Imaging Solutions, MI, USA). The mean number of positive cells per image area was determined.

**Statistical methods**

Statistical analysis was made using descriptive statistics, Mann–Whitney two-tailed unpaired tests, Kruskal–Wallis test, and negative binomial mixed regression (to account for multiple counts per sample). A P value <0.05 was considered as statistically significant. Statistical analysis was performed using the IBM SPSS Statistics for Windows (2016) (version 24.0; IBM Corp., Armonk, NY, USA and GraphPad Software, San Diego, CA, USA).

**RESULTS**

**Controls**

The positive controls for all markers indicated specific protein expression, whereas there was no specific immunostaining for the negative control sections (Figs. 1–4).

**CD34 and CD146**

The pulp vasculature in all regions of immature and mature teeth was positive for CD34 and CD146, and there was a homogeneous staining pattern from the capillaries to the larger blood vessels. There was no significant difference between anti-CD34 and anti-CD146 for identifying endothelial cells (P=0.50) in immature and mature permanent teeth. Depending on the plane of section, staining was detected on the endothelial cells of the vessels with a distinct lumen, as well as single and clusters of cells devoid of a lumen (Figs. 1 and 2).

Both tooth types showed spatial differences in the distribution, orientation, and size of the blood vessels. The coronal pulp was most vascular for immature and mature teeth (Table 1) with numerous small vessels in the CRZ, especially in the region of the pulp horns, and larger round vessels in the central pulp (Figs. 1 and 2a, d). The vessels in mature teeth tended to be larger and often had thickened walls. In the middle region of both tooth types, the blood vessels appeared larger and move consistently in an axial direction (Figs. 1 and 2b, e). There was very little pulp tissue present for the examination in the apical region of mature teeth, and samples were variable. Some specimens contained few blood vessels, whereas others showed numerous small immunopositive vessels (Figs. 1 and 2f). In immature teeth, the CRZ between the pulp and the apical papilla was richly vascular, containing a dense arrangement of small round blood vessels, whereas the apical papilla tissue was largely avascular with a few isolated vessels located centrally (Fig. 1c). Several sections showed isolated CD146+ single cells that were not associated with a lumen (Fig. 2c). At the periphery, in association with the periodontal ligament and residual dental follicle tissue, clusters of small immunopositive vessels were present.

Immature teeth had a similar vascular arrangement to mature teeth, but there were a greater MVD and more positive endothelial cells in teeth with incomplete root development (Table 1). Mann–Whitney tests showed that there were signifi-
Counting of CD34+ and CD146+ cells indicated that there were significantly more positive endothelial cells in the coronal (CD34 $P<0.001$ and CD146 $P<0.001$) and middle (CD34 $P=0.03$ and CD146 $P=0.001$) regions of immature teeth than those of mature teeth. Few positive cells were present in the apical papilla of immature teeth.

VEGF and VEGFR2

VEGF and VEGFR2 activities were present in the dentine–pulp complex of immature and mature teeth. There were more significantly more blood vessels in the coronal region of immature teeth than those of mature teeth ($P=0.03$), but there was no difference between the two tooth types in the middle region.

Spatial differences in expression were apparent within the pulps of immature teeth. Kruskal–Wallis statistics found significant differences in MVD between the coronal, middle, and apical papilla ($P<0.001$). Negative binomial mixed regression modeling indicated that the proportion of blood vessels in the middle was 0.72 times the proportion in the crown ($P=0.002$), and the proportion of vessels in the apical papilla was 0.27 times the crown ($P<0.001$). A similar pattern was observed in mature teeth where there was a significantly greater MVD in the coronal region than in the middle region ($P=0.04$), and regression analysis showed that there was a significantly greater proportion of blood vessels in the coronal pulp (1.32 times) than in the middle region ($P=0.03$).
TABLE 1. MVD and endothelial cell expression per 0.45 mm² for anti-CD34 and anti-CD146 in immature and mature permanent teeth (400×magnification)

<table>
<thead>
<tr>
<th></th>
<th>Immature teeth Mean (SD)</th>
<th>Mature teeth Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coronal</td>
<td>Middle</td>
</tr>
<tr>
<td>MVD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Maximum</td>
<td>37</td>
<td>21</td>
</tr>
<tr>
<td>CD34</td>
<td>62.0 (18.3)</td>
<td>45.0 (15.2)</td>
</tr>
<tr>
<td>Minimum</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td>Maximum</td>
<td>98</td>
<td>82</td>
</tr>
<tr>
<td>CD146</td>
<td>62.2 (20.1)</td>
<td>47.4 (17.0)</td>
</tr>
<tr>
<td>Minimum</td>
<td>37</td>
<td>14</td>
</tr>
<tr>
<td>Maximum</td>
<td>98</td>
<td>75</td>
</tr>
</tbody>
</table>

MVD, microvessel density; SD, standard deviation

TABLE 2. Cellular expression per 0.45 mm² for anti-VEGF and anti-VEGFR2 in pulp and apical papilla tissue of immature permanent teeth and pulp tissue of mature permanent teeth (400×magnification)

<table>
<thead>
<tr>
<th></th>
<th>Immature teeth Mean (SD)</th>
<th>Mature teeth Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coronal</td>
<td>Middle</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>13.0 (8.7)</td>
<td>7.1 (3.7)</td>
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<tr>
<td>Minimum</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Maximum</td>
<td>31</td>
<td>16</td>
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<tr>
<td>Non-endothelial cells</td>
<td>20.8 (12.6)</td>
<td>15.2 (14.4)</td>
</tr>
<tr>
<td>Minimum</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>50</td>
<td>51</td>
</tr>
<tr>
<td>Total positive cells</td>
<td>33.8 (19.2)</td>
<td>22.3 (15.5)</td>
</tr>
<tr>
<td>Minimum</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Maximum</td>
<td>81</td>
<td>61</td>
</tr>
<tr>
<td>VEGFR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>8.1 (5.6)</td>
<td>4.4 (3.4)</td>
</tr>
<tr>
<td>Minimum</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Maximum</td>
<td>20</td>
<td>13</td>
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<tr>
<td>Non-endothelial cells</td>
<td>17.8 (10.3)</td>
<td>5.8 (3.5)</td>
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<td>Minimum</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Maximum</td>
<td>37</td>
<td>12</td>
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<tr>
<td>Total positive cells</td>
<td>26.0 (12.84)</td>
<td>10.13 (4.5)</td>
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<tr>
<td>Minimum</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Maximum</td>
<td>51</td>
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</table>

VEGF, vascular endothelial growth factor; SD, standard deviation

VEGF+ cells in the pulp and apical papilla of teeth with open apices (Table 2). VEGF was expressed in the dentine matrix, the cytoplasm of some endothelial cells, and most blood vessels but also seen in non-endothelial cells (Table 2). There were significantly more VEGF+ cells in the coronal pulp of immature teeth than those of mature teeth (P=0.04), but there was no significant difference in the expression in the middle regions (P=0.27).

There were spatial differences in staining for VEGF throughout the pulps of immature and mature teeth, and angiogenic activity was present in the apical papilla (Figs. 3a–f and 4a–d). Mann–Whitney tests found that the pulps in both tooth types had significantly greater VEGF expression in the coronal region than that in the middle region (immature teeth P=0.04 and mature teeth P=0.02). The apical papilla contained few endothelial cells, but VEGF was expressed extracellularly on the cells unassociated with a lumen in immature teeth (Fig. 3f). The total VEGF expression in the apical papilla was not significantly different from the total amount of protein observed in the coronal pulp (P=0.17).

VEGF was consistently observed within the dentine matrix of immature and mature teeth (Figs. 3a–c and 4a, b). Staining intensity in the coronal region was ++ in all cases. Within the middle region, the intensity was ++ in 90% of immature teeth compared with 60% of mature teeth. The remaining 40% had a dentine matrix staining intensity of + that was also observed in the apical region for both tooth types. There was no staining at the terminal root apex.

The expression of VEGFR2 in the pulp and apical papilla was less than that of VEGF in all samples (Table 2). The receptor was present in a similar spatial arrangement to VEGF in the coronal, middle, and apical regions (Figs. 3g–i and 4e, f). However, VEGFR2 was not expressed within the dentine matrix or on odontoblasts. Some endothelial cells expressed VEGFR2, but not all blood vessels were positive. Although there was vari-
ability between samples, there was a significantly greater expression in immature teeth than that in mature teeth (P=0.001). For both tooth types, VEGFR2+ cells were seen in the CRZ, particularly in the region of the pulp horns, but there was a significantly greater expression in the coronal region of immature teeth than that of mature teeth (P=0.001). There were similar numbers of VEGFR2+ pulp cells in the middle region of both tooth types. VEGFR2 was expressed in the apical CRZ and the apical papilla where the expression was approximately half of that of the coronal pulp in immature teeth (Table 2).

**DISCUSSION**

The anatomical arrangement of the vascular system and the importance of endothelial cells within the pulp have been well described (19,20). It is accepted that teeth with open apices are associated with improved healing outcomes owing to a large pulp space and close proximity to the main vascular supply. However, recent studies demonstrated healing in the pulps of mature teeth following vital pulp therapy, and there is greater recognition of the importance of healthy pulp tissue, dental materials, cytokines, and angiogenesis in promoting the growth of new blood vessels for root development and pulp healing (21–23). Healthy tissue is necessary for healing. The present study has extended knowledge related to the spatial arrangement of the pulp vasculature and the distribution of key angiogenic factors within the dentine–pulp complex of teeth at different stages of root development. There is a greater vascularity in the pulps of immature permanent teeth, and the largely avascular apical papilla is adjacent to a vascular CRZ and dental follicle.

Morphological variability in the apical region is a recognized feature of mature teeth, and this limited the ability to draw conclusions about the vascularity and VEGF/VEGFR2 expression at the fully developed root-end (24). This region frequently contains anatomical anomalies and has a reduced pulp space with the deposition of secondary dentine that may explain in part the different clinical outcomes observed following injury. Gene expression studies of the apical pulp tissue from mature teeth are needed, but variability in pulp canal space and extraction of sufficient tissue for high quality RNA is challenging.

The ability of the dental pulp to respond to trauma with healing is determined by the local environment, but there is also a decline in its regenerative potential and vascularity with increasing age (20,25,26). While the findings from the present study indicate that healthy mature teeth have a lower MVD and fewer endothelial cells than immature teeth, the presence of VEGF and VEGFR2 in other studies in the dentine–pulp complex suggests that with appropriate signals, the tissue may still exert an angiogenic response. All mature tooth samples were obtained from participants of a similar age so it is unknown how pronounced these findings could be in an older age group and with continued physiological secondary dentine deposition.

Previous studies have demonstrated the presence of CD34 and CD146 and VEGF and VEGFR2 in the dental pulp (11, 13, 15, 20). However, it is not clear if clinical observations of pulp healing are related to stage of root development or spatial differences in blood vessels and VEGF/VEGFR2 expression. The increased vascularity and angiogenic activity observed in the coronal pulp compared with the radicular pulp for both tooth types may in part explain the favourable healing that frequently follows stepwise caries excavation and direct pulp capping (23,27). Moreover, although clinical factors, including an accurate diagnosis of pulp health and careful case selection, are important predictors of healing, in the present study, the large ranges in immunopositivity have been observed by others and aid our understanding of why some pulps heal and others do not (11).

CD34 and CD146 were both accurate in identifying the endothelial cells lining the blood vessels, and the use of two markers increased the understanding of the functional role of these cells in the pulp. CD34 is a cell surface protein that has an important role in vasculogenesis and pulp homeostasis (20) and is expressed by hematopoietic progenitor cells and on endothelial cell membranes. CD146 expression by endothelial cells indicated the potential to contribute to leukocyte–endothelium interactions as part of healing (26). The plane of section frequently results in ambiguity when examining staining. However, consistent with the findings by Trubiani et al. (20), we observed small clusters of CD34+ cells that may have been hematopoietic progenitor cells or cells in the process of vessel development. Fewer blood vessels and CD34+ cells were identified in the subodontoblast region of mature teeth, which may reflect pulp and vascular maturation and partially explain why pulp healing following injury to teeth with closed apices is often poor.

The findings of the present study confirm VEGF expression in the dentine–pulp complex (11, 13, 28) and extend this knowledge to show that there are spatial differences in the distribution of VEGF and VEGFR2 proteins in immature and mature teeth. Similar methodology and analysis was used by Artese et al. (11) and identified VEGF positivity in the blood vessels, the cytoplasm of fibroblasts, and inflammatory cells, as well as some odontoblasts. This may indicate that when there is injury to a healthy pulp VEGF may exert different angiogenic effects and several cell types are involved. The expression of VEGF and VEGFR2 in the apical region of immature teeth and distinct from blood vessels indicates the potential of the tissue to facilitate angiogenesis when stimulated (29). The apical papilla of immature teeth contains a unique stem cell niche and cells that are able to show an angiogenic response, including an up-regulation of VEGF receptor genes (6). Therefore, although the papilla is largely avascular, the presence of protein in this region means that the tissue might contribute to angiogenesis independent of vascularity.

While the importance of VEGF in angiogenesis following pulp injury is becoming clearer, there is a limited understanding of the role VEGFR2 may have in this process (15,30). Until now, differences in protein expression between immature and mature teeth have not been identified. In contrast to the findings by Grando Mattuella et al. (15) who used immunohistochemistry to examine primary and permanent teeth with closed apices, we found that mature teeth showed variable VEGFR2 expression in the pulp. VEGFR2 was consistently observed in
teeth with open apices, particularly in the apical papilla where it is available to bind to VEGF. These findings support the growing evidence around the functional importance of VEGFR2 in immature teeth for pulp healing, root development, and maturation. Further studies at a molecular level are warranted to investigate VEGF/VEGFR2 signaling in mounting an angiogenic response to inflammation.

CONCLUSION

In conclusion, there were spatial differences in the expression of vascular and angiogenic (VEGF/VEGFR2) markers in healthy immature and mature permanent teeth. Immature permanent teeth had a greater vascularity and angiogenic potential than teeth with complete root development, but the coronal pulp of both showed the greatest MVD and angiogenic protein expression that may contribute to favourable healing observed following some pulp injuries. VEGF/VEGFR2 expression was evident throughout the dentine–pulp complex, within the dentine matrix, the pulp, and the apical papilla. Spatial differences in expression suggest variability in the angiogenic potential in different regions of the pulp, and this process may be independent of the vascular arrangement.

Disclosures

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Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethics Committee Approval: All procedures were in compliance with the Lower South Regional Ethics Committee, New Zealand (Project no.: LRS/08/06/028).

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