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Pulpal status of human primary molars with co-existing caries and physiological root resorption

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Running title: Pulp status of human primary molars

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Summary

Aim. This study sought to investigate the effect of caries, in association with physiological root resorption, on the pulpal status of human primary molars.

Design. Fifty three mandibular primary molars were obtained from children requiring extractions under general anaesthesia. Following extraction, teeth were split longitudinally and placed in Zamboni's fixative. Teeth were categorised according to i) the depth of caries (less than or greater than half way through dentine thickness) and ii) the degree of physiological root resorption (less than 33%, 34-66% or greater than 67% of the root length). 10µm pulp sections were subject to indirect immunofluorescence using a combination of PGP 9.5 (a general neuronal marker), CD45 (a general neuronal marker) and *Ulex europaeus* agglutinin I (a marker of vascular endothelium). Image analysis was used to determine the percentage area of staining (PAS) for innervation and immune cells.

Results. Marked differences were seen between different samples but there were no significant differences in mean PAS for PGP 9.5 or CD45 according to the degree of caries or extent of physiological root resorption (two-way ANOVA, $p>0.05$).

Conclusion. Findings suggest that even if primary molars are undergoing exfoliation they show comparable caries-induced changes to teeth without physiological root resorption, thus retaining potential for healing and repair.

Introduction

Basic science research is increasingly recognising the tooth's inherent defence and repair mechanisms, and this knowledge is being translated into primary pulp therapy regimens. Over the last decade, there has been a shift away from invasive procedures and non-biologically compatible materials towards indirect pulp therapies, which aim to preserve pulp vitality and stimulate new hard tissue formation[1].

Although studies have shown that carious primary teeth have a similar potential for healing and repair as the permanent dentition[2,3], the co-existence of the natural process of exfoliation in the primary tooth presents a possible modifying effect. Indeed, the extent to which root resorption may impact the pulp's defence mechanisms is largely unknown. A number of studies have, however, explored changes in the pulpal anatomy of healthy resorbing primary teeth. Mohiuddin's early histological studies revealed degenerative neural changes in primary teeth just before the onset of visible root resorption[4]. These changes included varicosities, vesicular formation and fragmentation within nerve fibres, which became more pronounced with advancing resorption. Interestingly, subsequent research found that degenerative neural changes were actually observed in primary, and indeed permanent teeth, in the absence of any active root resorption[5]. There does, however, appear to be consensus that the process of exfoliation is associated with an increase in immune cells within the tooth pulp, and the presence of odontoclasts is also reported at advanced stages[6-8]. Angelova and co-workers examined 43 healthy primary teeth with physiological root resorption[9]. Teeth were categorised into four groups according to degree of root resorption and a number of lymphocyte sub-populations were quantified. The key finding was that immunocompetent cells increased with physiological root resorption, which was suggestive of an altered immunocompetency within the tooth pulp. Other

authors have reported that the dental pulp remains in a relatively 'normal' condition until the root resorption level has advance to approximately 1mm below the cement-enamel junction. It is only at this stage that chronic inflammatory cells (B and T lymphocytes) infiltrate the coronal pulp which, along with bacterial ingress through the gingiva-dental junction, accounts for the dense accumulation of inflammatory cells during the final stages of exfoliation[10].

In order to remove the potential confounding effects of physiological root resorption on caries-related pulpal inflammation and vice versa, investigators have tended to restrict their experimental material either to non-carious primary teeth with physiological root[11] or to carious primary teeth without any root resorption[2-3]. Having only gained insight into the individual effects of caries or physiological root resorption on pulpal status, the clinical applicability of these findings was limited. In the real life setting, physiological root resorption is likely to have commenced in children over the age of six years, thus clinicians will be undertaking restorative or vital pulp therapies in carious teeth with co-existing root resorption.

Therefore, this study aimed to explore both variables: the research hypothesis being that there are no differences in the degree of caries-induced inflammation in human primary molars according to the co-existence of any physiological root resorption.

Materials and Method

Experimental material

The experimental material comprised extracted carious mandibular second primary molars. These were obtained from fit and healthy children who required multiple dental extractions under general anaesthesia (GA) at a UK dental hospital during the period July 2010 to April 2011. These were children who were not able to cope with treatment under local

anaesthetic or sedation. There was no facility to restore carious primary teeth under GA on this particular operating list. Thus potentially restorable carious primary molars were removed in some high caries risk and pre-cooperative children requiring an urgent GA, to ensure they had no need of further immediate treatment and to reduce the risk of a repeat GA. Teeth with clinical or radiographic signs of loss of vitality were not included in the sample.

Appropriate ethical approval for the study was obtained (Ref: 10/H1306/91) and written parental consent and child assent were obtained prior to tooth collection.

Root measurement

Following forceps extraction, the mesial and distal roots were examined closely for any visible resorption and the root with the greatest degree of resorption was selected for subsequent measurement. The distance between the cemento-enamel junction and the first point of visible root resorption (if there was evidence of this) was recorded using an electronic millimetre calliper (Digimatic Calliper, Mitutoyo, UK, Ltd) by one investigator. The percentage of the total root length that had undergone any resorption could then calculated using Kramer and Ireland's normative data[12], where an intact mesial root was taken to be 11.37 mm and an intact distal root taken to be 10.55 mm. The following working example is given to show how the percentage of root resorption would be calculated for a mesial root with a distance of 7 mm remaining (ie root length unaffected by resorption) between the cemento-enamel junction and the first point of any root resorption.

$$\text{Percentage of root undergone resorption} = \frac{(11.37-7) \times 100\%}{11.37}$$

=38.4%

After determining the amount of root resorption by the above method, teeth were allocated into one of the three subcategories to simplify future analysis. Group 1 comprised teeth with less than 33% of their root length affected by resorption; group 2 had between 34% and 66% of their root length affected by resorption and group 3 had greater than 67% of their root length affected by resorption.

Tissue preparation

Teeth were then split longitudinally through the centre of the carious lesion. For teeth with occlusal caries, a superficial vertical groove was first cut on the buccal surface using a fine diamond bur. For teeth with proximal caries, a vertical groove was cut on the proximal surface. A 5mm osteotome was then placed in the groove and struck with a surgical mallet to split the tooth into half. These halves were immediately immersed in Zamboni's fixative (4% paraformaldehyde, 0.2% picric acid, 0.1M PBS) and kept at 4°C. The Zamboni's fixative was replaced with 0.1M phosphate buffered saline (PBS) after 24 hours and stored for up to two weeks at 4°C.

The coronal pulp tissue was dissected out from the pulp chamber with a small dental excavator and surgical scalpel under a Nikon dissection microscope at x10 magnification. The coronal pulps were stored in 0.1M PBS containing 30% sucrose solution for cryoprotection (5h at 4°C) and embedded in Tissue-tek OCT compound (Bayer Diagnostics, Basingstoke, UK). Longitudinal sections were cut at 10µm using a microtome cryostat (Microm HM 500 OM, Waldorf, Germany) and were collected on poly-D-lysine-coated glass slides (Sigma, Poole, UK).

After sectioning, the slides were left at room temperature for 60 minutes to air dry and were then stored at -80°C prior to immunostaining.

Immunostaining was performed using an indirect immunofluorescence method and previously established protocols[2,3]. Slides were first washed in PBS containing 0.2% Triton X-100 (Bayer Diagnostics, Basingstoke, UK), and then incubated in PBS and triton containing 10% normal goat serum (Vector Laboratories, Peterborough, UK) for 30 minutes at room temperature. Following this, sections were triple-labelled using a mixture of:

1. a polyclonal antibody to protein gene product 9.5 (PGP 9.5), a general neuronal marker (rabbit anti-human PGP 9.5, dilution 1:2000, Ultraclone, Isle of White, UK);
2. a monoclonal antibody to leukocyte common antigen (CD45), a universal marker for leukocytes (mouse anti-human LCA, dilution 1:1000, Dako, Bucks, UK); and
3. biotinylated *Ulex europaeus* agglutinin I (UEAI), a marker of human vascular endothelium (dilution 1:100, Vector, Vector Laboratories, Peterborough, UK).

The antisera and UEAI were diluted in PBST containing 5% normal goat serum, and sections were incubated for 24 hours at 4°C. Before incubating, slides were then washed again in PBS (2 x 10 minutes) for a further 90 minutes at room temperature using a mixture of fluorescent secondary antibodies:

1. goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (dilution 1:50, Vector);
2. horse anti-mouse IgG conjugated to Texas red (dilution1:100; Vector); and
3. 7-amino-4-methyl-coumarin-3-acetic acid-conjugated streptavidin (dilution 1:25, Vector).

The fluorescent labels were diluted in PBS and triton containing 2% normal goat serum. Slides were finally washed again in PBS (2 x 10 minutes) before mounting with Vectashield (Vector). Immunohistochemical controls for PGP 9.5 and CD45 were performed by incubating sections with the antibody diluent alone. The specificity of the EUAI reaction was tested by inhibiting lectin binding with the use of 0.2 M a-L-fucose (Vector) dissolved in PBS containing 0.2% PBST. No positive labelling was seen in any of the controls.

Image analysis

Sections were viewed using a Zeiss axioplan fluorescent microscope with the x10, x20 and x40 objectives in order to systematically examine tooth pulp sections for overall anatomical features. The region of tooth pulp estimated to be directly below the carious lesion (determined by referring to stored photographic images of the tooth half) was then subject to quantitative analysis using the x20 objective and previously described methodology. Computer-assisted image-analysis software (Image-Pro Plus v3.0; Media Cybernetics, Silver Spring, MD, USA) was used to create a digital image from the microscopic image. The percentage area of staining (PAS) for PGP 9.5-, CD45-, and UEAI-labelled tissue was then automatically determined within the 0.22mm^2 field of analysis.

Caries assessment

After pulp tissues had been dissected out for immunocytochemistry analysis, each remaining sectioned tooth half was photographed using a Nikon D300 digital camera (105mm lens, AF micro nikkor 1:2.8D), preset stage distances and an external light source. Subsequently,

the high quality digital pictures were printed onto uniform photographic paper size of 13.5mm x 20.5mm using an Epson Stylus printer 1400 to minimise variability.

A clinical assessment of degree of caries was made on the basis of colour changes by one investigator using these standardised photographs. Each sectioned tooth sample with the deepest clinical extent of caries was simply categorised into one of three subgroups as follows: group 1=no caries/enamel caries only; group 2=caries involving less than half the dentine thickness; group 3=caries extending greater than half way through the dentine thickness.

Repeatability

Intra-examiner repeatability for measurements relating to root length and the percentage area of staining (PAS) for innervation density was determined by repeating the measurements on 10% of the samples one week after the initial examination. The mean percentage of difference and levels of agreement were calculated using Bland and Altman's statistical model and plots[13]. Intra-examiner repeatability for caries subgroups was also assessed by repeating the categorisation on 10% of the sample and calculating a kappa coefficient for the agreement between the two assessments.

Statistical analysis

Two-way ANOVA was used to test for statistically significant differences in PAS of nerves and immune cells according to the two independent variables: degree of root resorption and degree of caries. Where appropriate, this was followed by Tukey's test for multiple pairwise comparisons of means in order to determine whether there were any significant differences between specific subgroups. All statistical analyses were performed on logarithmically

transformed (log10) data, as preliminary analysis revealed that the data were not normally distributed. The significance levels were set at $p<0.05$.

Results

Experimental material

Immunocytochemical analysis was undertaken on 53 mandibular primary second molars. Tooth samples were collected from 52 children, who had a mean age of 5.7 years (SD± 1.65, range=3-9 years). Table 1 shows sample numbers within each of the three caries subgroups and their distribution according to the degree of physiological root resorption. It can be seen that there were only two samples in caries group 1, thus caries groups 1 and 2 were combined for subsequent statistical analysis.

Intra-examiner repeatability

For categorisation of the degree of caries, there was intra-examiner agreement for 85% of sample and an un-weighted kappa value of $\kappa=0.8$, indicating substantial agreement[14]. The Bland Altman agreement limits for root resorption and PAS for neural innervation were found to be -0.015% (± 3.52) and -0.15% (± 0.56) respectively, again confirming good intra-examiner repeatability.

Immunocytochemical analysis

Qualitative analysis revealed that PGP 9.5-immunoreactive (IR) nerve fibers were present in all pulp sections and throughout the coronal pulp. As described in numerous previous studies[2,3] fine, beaded PGP 9.5-IR fibres were clearly visible within the pulp periphery and were seen to extend into the odontoblast layer. In the mid-coronal region, PGP 9.5-IR fibres were

predominantly present in thick nerve trunks, or neurovascular bundles. Some samples showed a marked increased density of nerve fibres within the pulp horn region and sub-odontoblastic plexus, which was apparent in both caries subgroups and at all stages of root resorption (Figure 1).

The immunostaining with CD45 revealed small rounded structures, with an appearance and size consistent with that of B lymphocytes. Again there were marked differences in the density of CD45-IR cells between different pulp samples, which seemed to show little correlation with the extent of caries or degree of physiological root resorption: in some sections there were isolated CD45-IR cells and in others, dense accumulations could be seen (Figure 2). Furthermore, there was no obvious relationship between an increase in CD45-IR tissue and an increase in PGP 9.5 IR tissue within individual samples.

Staining for UEAI was inconsistent, with good staining in some sections and very faint staining in others. Therefore, quantitative image analysis could not be reliably undertaken in this study.

Initial observation of the quantitative data for mean PAS for PGP 9.5-IR tissue suggested that neural innervation was greater in samples with caries extending greater than half way through the dentine for teeth with root resorption involving up to two thirds of the root length (Figure 3). However, using two-way ANOVA on transformed data revealed that there was no significant effect of the degree of caries (two subgroups) or extent of resorption (three subgroups).

Descriptive analysis of mean PAS for CD45-IR tissue revealed that immune cell accumulation was greatest for teeth with caries involving less than half the dentine and with less

than a third root resorption (Figure 4). However, two-way ANOVA on transformed data failed to show a significant difference according to the degree of caries or root resorption.

Discussion

This anatomical study examined changes in pulpal innervation and inflammation in human primary molars, which were subject to concurrent pathological (caries) and physiological (exfoliation) processes. A key finding was the extreme variation seen between tooth samples, even with similar degrees of caries involvement and physiological root resorption. It was noted that some teeth with gross caries actually had fewer immune cells than those with caries affecting less than half the dentine thickness. This observation prompts two important clinical questions: why is there such variability in pulpal responses to caries and how reliable is caries depth as a determinant of pulpal status?

Initial caries-induced pulpal injury comes from plaque acids, which solubilise the dentine matrix and diffuse through dentinal tubules. The dentine matrix has a buffering capacity and serves to lower the pH of the plaque acid. However, the relative strength of the acid reflects the rate and severity of the caries progression[15]. Differences in dentine permeability, size, length and mineral contents of dentinal tubules will all affect the rate of caries progression. It is also recognised that a variety of other host-related factors will modify caries effects on the pulp-dentine complex, contributing to the wide inter-individual biological variation seen in human studies[16]. Furthermore, the activity of the caries lesion itself may be the most important determinant in eliciting a pulpal response. Di Nicolo and colleagues undertook a histological study of 36 extracted carious primary molars and correlated pulpal status with caries activity[17]. They found that pulpal necrosis was significantly less likely under arrested lesions, compared to

active ones. However, accurate diagnosis of caries activity, in the clinical setting, still relies on subjective visual and tactile assessment as the use of aids, such as caries-detector dyes remain inconclusive[18]. Clearly, the depth of caries progression alone is not a reliable predictor of pulpal status, in the clinic or laboratory. However, this parameter was selected for the present study, as caries depth is widely considered in clinical decision-making. In the absence of patient-reported symptoms, clinicians frequently rely on intra-oral radiographs to reach a decision on how best to restore a carious tooth. Radiographic examination reveals the proximity of the carious lesion to the pulp chamber and thus the potential for pulpal sequelae[19].

A more robust predictor of pulpal status may be the remaining dentine thickness (RDT). This is defined as the minimum depth of dentine from the base of a carious lesion to the odontoblast layer, and studies have shown it to be an important factor in odontoblast survival and dentine repair. Murray and colleagues found that a remaining dentine thickness of less than 0.3mm following cavity preparation in permanent teeth was associated with persistent pulpitis[20]. Very few studies have assessed the correlation between RDT and pulpal status in primary teeth. However, one study did show that inflammatory changes are present within the primary tooth pulp when RDT is still at 1.8mm[21]. Considering that the average depth of occlusal enamel in primary molars is 2mm, caries-induced pulpal changes can occur even with minimal caries involvement, as shown in the present study. Therefore, to summarise the implications from the present study's first key finding, clinicians should be aware that there is considerable inter-individual variation in caries-induced pulpal inflammation in primary teeth and the depth of caries *per se* is not a reliable indicator of pulp status.

The second key finding from this study is that pulpal defence and healing mechanisms are apparent even when physiological root resorption has involved up to two thirds of the root

length. This was supported by frequent observations of profound neural branching and thickening in some carious samples. A wealth of research has indicated the role of pulpal nerves in mounting responses to tissue injury and repair by modifying blood flow and immune cell responses[22]. It is therefore proposed the carious primary molars with concurrent physiological resorption can evoke healing and repair responses. From a clinical point of view, this finding suggests that restorative interventions, using appropriate local analgesia are warranted for teeth even with advancing root resorption. Furthermore, in view of the anatomical changes seen, conservative biological approaches for the treatment of deep caries such as indirect pulp capping, stepwise excavation or sealing in of caries with preformed metal crowns (the Hall technique)[23] have a sound biological basis.

One has to be cautious, however, in drawing clinical conclusions from the findings of this and related studies as they stem from anatomical observations, rather than physiological experiments. In addition to the acknowledged variability in caries lesion activity, the activity of the resorption process may also have had a modifying effect on immune cell accumulation and innervation. Physiological root resorption in primary teeth is a dynamic and complex process, with periods of quiescence and activity as is the case for bony remodelling. Furthermore, as root resorption can affect the lateral aspect of the roots as well as the furcation region, the simple measurement of the amount of apical root resorption, as adopted in the present study, fails to capture the more subtle underlying biochemical and molecular changes.

To our knowledge there has only been one previous study exploring the co-existence of caries and root resorption on pulpal status. Simsek and Duruturk sought to quantify immune cell responses within the pulp tissue of 49 primary teeth with various degrees of caries and root resorption[24]. Caries depth was determined from standardised bite-wing radiographs and was

classified into three groups. Root resorption was measured using the methodology described by Kramer and Ireland and teeth were categorised into the same three subgroups as the present study [12]. Flow cytometry employed to determine the type and quantity of immunocompetent cells in the experimental subgroups. The researchers found a significant increase in some immune cell populations in association with caries progression and physiological root resorption, concluding that the primary tooth pulp was able to maintain its healing and defence capacity against advancing caries and progressive root resorption. Anatomical observations from the present study concur with those of Simsek and Duruturk. However, the present study did not reveal any statistically significant increases in immune cells, due to an inadequate sample size in some of the experimental subgroups.

Conclusion

Within the acknowledged limitations of a purely anatomical study, this study has demonstrated wide inter-individual variability in the innervation and immune cell status of carious primary molars, which are in the process of exfoliation. Findings lend further support for the healing potential of the primary tooth and the adoption of regenerative pulp therapies in the clinical setting.

Bullet points

Why this paper is important to paediatric dentists

- Findings from this study reveal that primary teeth, which are undergoing physiological root resorption, appear to retain their innervation and ability to mount an immune response to

caries progression. Vital pulp therapies and appropriate use of local anaesthetic are therefore still indicated for the management of resorbing carious primary teeth.

- Clinicians should be aware of the marked variation in pulpal innervation and inflammation in primary carious teeth: it is likely that the activity of the carious lesion and permeability of the dentine may be more predictive of the underlying pulpal inflammation than the remaining dentine thickness alone.

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Figures legends

Tables

Table 1. Sample numbers according to degree of caries and proportion of root affected by physiological resorption.

	Group 1	Group 2	Group 3	
Degree of root resorption	**No caries/enamel caries only**	**Caries extending to halfway through dentine thickness**	**Caries greater than halfway through dentine thickness**	**Total**
<33% of root length	*n* = 0	*n* = 9	*n* = 10	*n* = 19
34–66% of root length	*n* = 2	*n* = 15	*n* = 13	*n* = 30
>67% of root length	*n* = 0	*n* = 2	*n* = 2	*n* = 4
Total	*n* = 2	*n* = 26	*n* = 25	*n* = 53

Figures

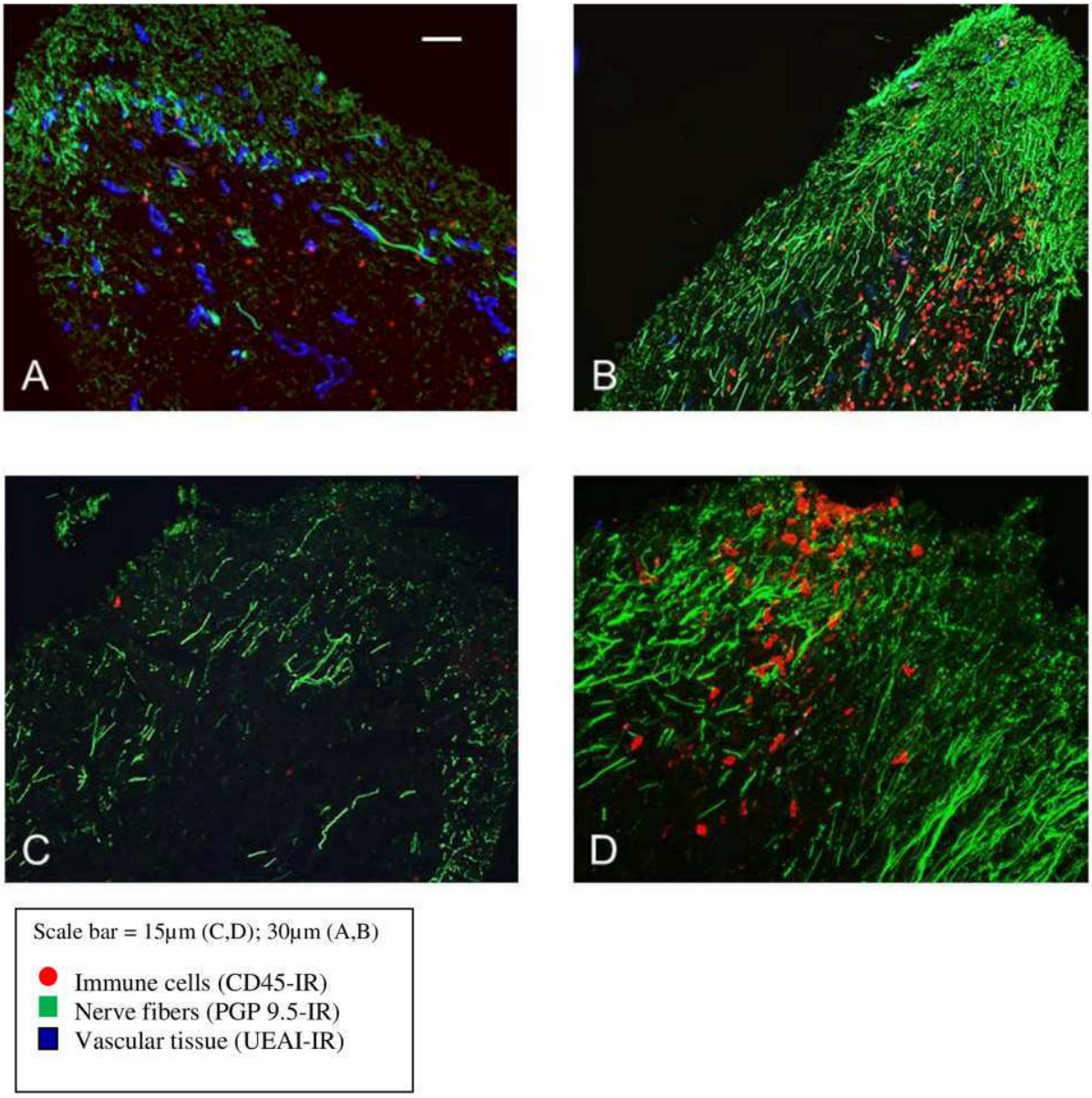

Figure 1. Photomicrographs selected to demonstrate differences in the distribution of PGP 9.5-immunoreacative (IR) nerve fibres in the pulp of human primary molars with varying degrees of caries and physiological root resorption. **(A)** Slight increase in the density of the subodontoblastic nerve plexus in the pulp horn of a tooth with caries extending greater than half way through the dentine thickness and less than 33% of its root subject to physiological root resorption. **(B)** Dense innervation throughout the pulp horn region of a tooth with caries extending greater than half way through the dentine thickness and with up to 66% of its root subject to physiological root resorption. **(C)** Normal, fine beaded peripheral nerve fibres in the

pulp horn of a tooth with caries less than half way through dentine and up to 66% of its root subject to physiological root resorption. **(D)** Thickening of nerve fibres and overall increased neural density in the pulp horn of a tooth with caries less than half way through dentine and with up to 66% of its root subject to physiological root resorption.

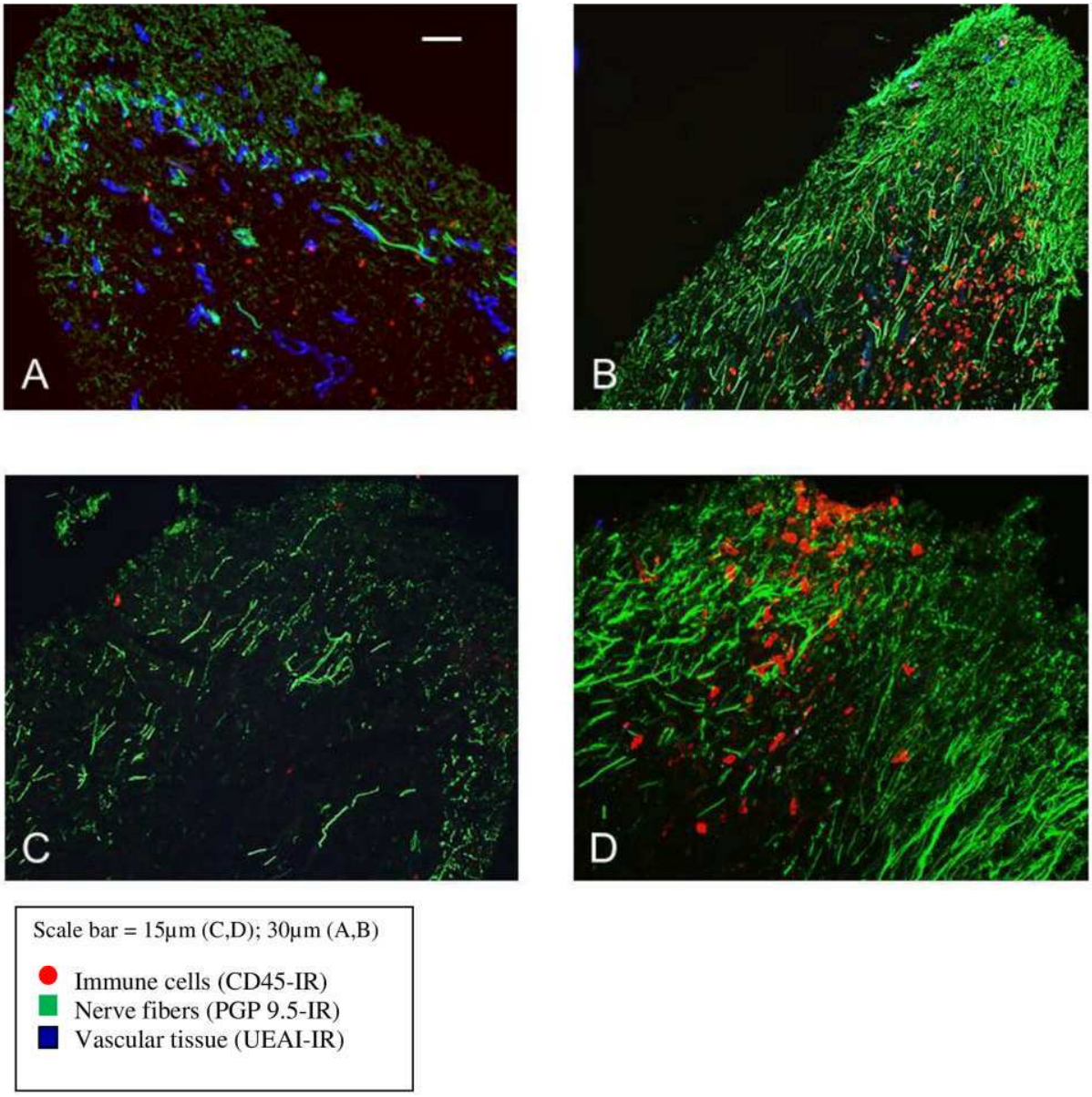

Figure 2. Photomicrographs selected to demonstrate differences in the distribution of CD45-immunoreacative (IR) immune cells in the pulp of human primary molars with varying degrees of caries and physiological root resorption. **(A)** Scattered sparse immune cells in the pulp horn of a tooth with caries extending less than half way through the dentine thickness and up to 66% of its root subject to physiological root resorption. **(B)** Focal accumulation of immune cells in the pulp horn region of a tooth with caries extending greater than half way through the dentine thickness and up to 66% of its root subject to physiological root resorption. **(C)** Increased

number of immune cells in the pulp horn of a tooth with caries greater than half way through dentine and less than 33% of its root subject to physiological root resorption. **(D)** Dense accumulation of immune cells in the pulp horn of a tooth with caries greater than half way through dentine and less than 33% of its root subject to physiological root resorption.

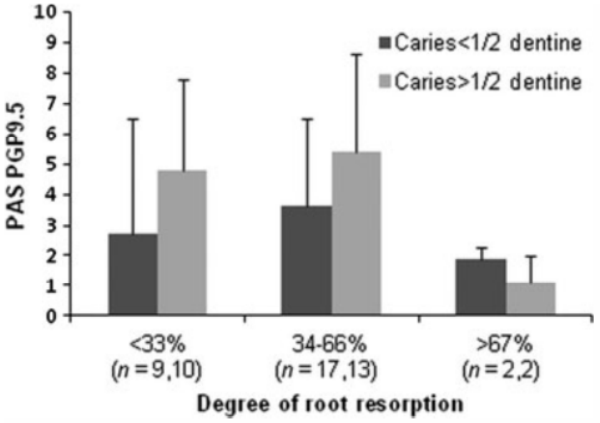

Figure 3. Mean percentage area of staining for innervation (PAS PGP 9.5) according to caries subgroup and percentage of root resorption for primary molar tooth pulps.

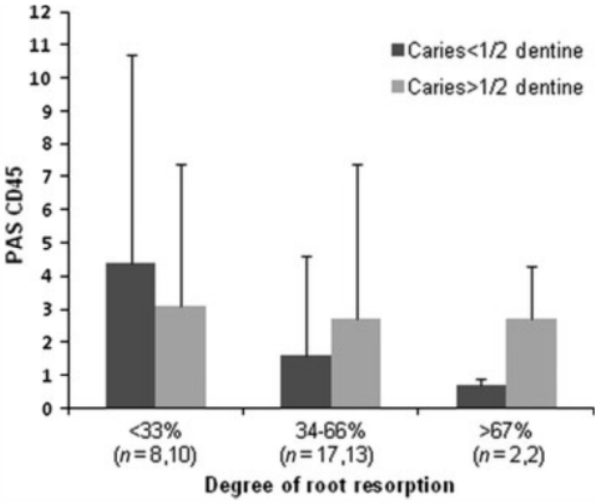

Figure 4. Mean percentage area of staining for immune cells (PAS CD45) according to caries subgroup and percentage of root resorption for primary molar tooth pulps.