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Pulpal status of human primary teeth with physiological root resorption

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Objective. The overall aim of this study was to determine whether any changes occur in the pulpal structure of human primary teeth in association with physiological root resorption.

Methods. The experimental material comprised 64 sound primary molars, obtained from children requiring routine dental extractions under general anaesthesia. Pulp sections were processed for indirect immunofluorescence using combinations of: (i) protein gene product 9.5 (a general neuronal marker); (ii) leucocyte common antigen CD45 (a general immune cell marker); and (iii) *Ulex europaeus* I lectin (a marker of vascular endothelium). Image analysis was then used to determine the percentage area of staining for each label within both the pulp horn and mid-coronal region. Following measurement of the greatest degree of root resorption in each sample, teeth were subdivided into three groups: those with physiological resorption involving less than one-third, one-third to two-thirds, and more than two-thirds of their root length.

Results. Wide variation was evident between different tooth samples with some resorbed teeth showing marked changes in pulpal histology. Decreased innervation density, increased immune cell accumulation, and increased vascularity were evident in some teeth with advanced root resorption. Analysis of pooled data, however, did not reveal any significant differences in mean percentage area of staining for any of these variables according to the three root resorption subgroups ($P > 0.05$, analysis of variance on transformed data).

Conclusions. This investigation has revealed some changes in pulpal status of human primary teeth with physiological root resorption. These were not, however, as profound as one may have anticipated. It is therefore speculated that teeth could retain the potential for sensation, healing, and repair until advanced stages of root resorption.

Introduction

Primary teeth have one very distinct feature which sets them apart from the permanent dentition: they undergo physiological resorption leading to shedding. This complex phenomenon is not yet fully understood and remains of considerable biological and clinical interest. It is generally agreed that consistency in the timing and pattern of primary root resorption, and subsequent permanent tooth eruption, are indicative of related and genetically programmed events[1].

Previous studies have identified the dental follicle and stellate reticulum of the permanent successor as having a key role in tooth resorption[2,3]. The dental follicle is thought to be responsible for recruitment of mononuclear cells and providing a favourable environment for their differentiation into osteoclasts[1,4]. These multinuclear giant cells adhere to bone and initiate resorption via acidification of the extracellular matrix[5]. The cells actually responsible for dental tissue resorption are odontoclasts, which appear to belong to the same cell line as osteoclasts[5,6]. Cytokine-producing cells, capable of mediating odontoclast activity, have recently been identified within the pulp tissue of primary teeth[7]. This finding supports the role of the primary tooth pulp, as well as that of the developing permanent successor, in the resorption process.

The focus of this paper, however, is not to review the mechanisms of physiological resorption, but to consider whether there are any changes in pulp histology concurrent with

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this unique process. Without this basic science insight, it is difficult for clinicians to make informed treatment decisions when managing the primary dentition. Knowledge of a tooth's potential to respond to injury, according to the stage of root resorption, would be invaluable in predicting the likely success of interventions such as indirect pulp capping or vital pulpotomy.

In terms of overall histology, it has been reported that primary teeth maintain a similar pulpal architecture to that seen in young permanent teeth, at least until very advanced resorption[8]. Furthermore, there appear to be no gross histological differences between primary pulps at different stages of resorption[9]. The anatomical characteristics of odontoblast cells also seem unaffected during tooth shedding[9,10].

In recent years, investigators have sought a more detailed assessment of pulp status during the resorptive process, particularly in relation to immune cell number and type. There is general consensus that the overall number of pulpal inflammatory cells increases from the beginning of the resorption process until exfoliation[8,11–14]. With respect to changes in specific immune cell populations, studies have reported increases in macrophages as well as T and B lymphocytes with advanced root resorption[15,16]. Interestingly, Simsek and Durutürk's study of both carious and sound primary teeth, found that, for non-carious teeth, only natural killer cells significantly increased with resorption[16].

Knowledge of any resorption-related changes in other important pulp structures, namely nerves and blood vessels, is more rudimentary. Rapp and coworkers examined pulpal innervation in 75 human primary teeth at different stages of resorption, using a silver impregnation technique[17]. A similar pattern of innervation was observed in all samples. With advanced resorption, however, neural thickening (varicosities), fragmentation, and reduced innervation density were reported. A further subjective observation was decreased density of the subodontoblastic nerve plexus, with fewer fibres extending into the odontoblast cell layer.

Findings from descriptive studies of pulpal vascularity are conflicting. Sari and colleagues examined 14 extracted primary canines at different stages of root resorption using haematoxylin and eosin staining, and light microscopy[9]. They reported normal pulpal vascularity in resorbed samples. In contrast, a more recent study of 19 primary teeth with physiological root resorption, also using haematoxylin and eosin staining and light microscopy, identified hyperaemia and dilated blood vessels in a small number of teeth[14].

Overall, there would appear to be a paucity of literature exploring changes in the pulpal structure of primary teeth with physiological root resorption. Therefore, the aim of this study was to undertake a comprehensive quantitative investigation of pulpal innervation density, immune cell accumulation, and vascularity in human non-carious primary teeth with variable degrees of root resorption.

Materials and methods

Experimental material

Maxillary and mandibular first and second primary molars comprised the experimental material for the study. Inclusion criteria dictated that tooth samples were: caries free or had minimal enamel caries only, had a permanent successor, had no enamel defect or excessive tooth tissue loss, and did not sustain a root fracture during extraction.

Teeth were obtained from fit and healthy children who required routine dental extractions under general anaesthesia (GA) at the day-care unit of Leeds Dental Institute, UK. Treatment plans were prescribed at a pre-GA assessment by a consultant paediatric dentist. Teeth were collected by one investigator (J.M.) during a 4-month period (September to November 2007). Ethical approval was granted by the Leeds (West) Research Ethics Committee, and informed consent was obtained from legal guardians, prior to the GA, to allow the use of their child's extracted teeth for the specific purposes of this research.

Tissue preparation

Immediately following forceps extraction, a longitudinal groove was cut on the buccal aspect of the tooth, using a diamond disc. The

tooth was then split longitudinally by placing an osteotome in the buccal groove and applying a blow with a surgical mallet. Tooth halves were placed in fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4) for 24 h at 4 °C. The coronal pulp was then carefully removed and placed in phosphate-buffered saline (PBS). Coronal pulps were left in PBS for 24 h at 4 °C before placing in 0.1 M PBS containing 30% sucrose solution for cryoprotection (5 h at 4 °C). The pulp tissue was then embedded in Tissue-Tek OCT compound (Bayer Diagnostics, Basingstoke, UK), and 14 μm longitudinal sections were cut from each tooth pulp and collected on poly D-lysine-coated glass slides (Sigma, Poole, UK). Fifteen sections were collected from each tooth pulp.

Immunocytochemistry and lectin histochemistry

Immunostaining was performed using an indirect immunofluorescence method[18]. Slides were first washed in PBS containing 0.2% Triton X-100 (PBST) (2 × 10 min) and then incubated in PBST containing 10% normal goat serum (Vector Laboratories, Peterborough, UK) for 30 min at room temperature. Following this, sections were triple-labelled using a mixture of: (i) a monoclonal antibody to protein gene product 9.5 (PGP 9.5) a general neuronal marker (rabbit antihuman PGP 9.5, dilution 1 : 1000, Ultraclone, Isle of White, UK); (ii) a monoclonal antibody to leucocyte common antigen (LCA) – a universal marker for leucocytes (mouse antihuman LCA, dilution 1 : 100, Dako, Bucks, UK); and (iii) biotinylated *Ulex europaeus* agglutinin I lectin (UEIL) – a marker of human vascular endothelium (dilution 20 μg/mL, Vector).

The antisera and UEIL were diluted in PBST containing 5% normal goat serum, and sections were incubated for 24 h at 4 °C.

Slides were then washed again in PBS (2 × 10 min) before incubating, for a further 90 min at room temperature, with a mixture of fluorescent secondary antibodies: goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (dilution 1 : 20, Vector), horse anti-mouse IgG conjugated to Texas red (dilution 1 : 100; Vector), and 7-amino-4-methyl-3-acetic acid-conjugated streptavidin (dilution 1 : 25, Vector). The fluorescent labels were diluted in PBST containing 2% normal goat serum. Slides were finally washed again in PBS (2 × 10 min) before mounting with Vectashield (Vector).

Immunohistochemical controls for PGP 9.5 and LCA were performed by incubating sections with the antibody diluent alone. The specificity of the lectin reaction was tested by inhibiting lectin binding with the use of 0.2 M α-L-fucose (Vector) dissolved in PBS containing 0.2% PBST. No positive labelling was seen in any of the controls.

Analysis of immunolabelling

Sections were viewed using a Zeiss (Oberkochen, Germany) axioplan fluorescent microscope, and all analyses were performed blind. Two different fields were subject to quantitative analysis: the mesio-buccal pulp horn, as it receives the greatest proportion of nerve terminals, and the mid-coronal pulp region which contains large blood vessels and nerve trunks (Fig. 1).

The method used to quantify labelling has been described previously[19–21]. Essentially, computer-assisted image analysis software (Image-Pro Plus v3.0; Media Cybernetics, Silver Spring, MD, USA) was used to create a digital image

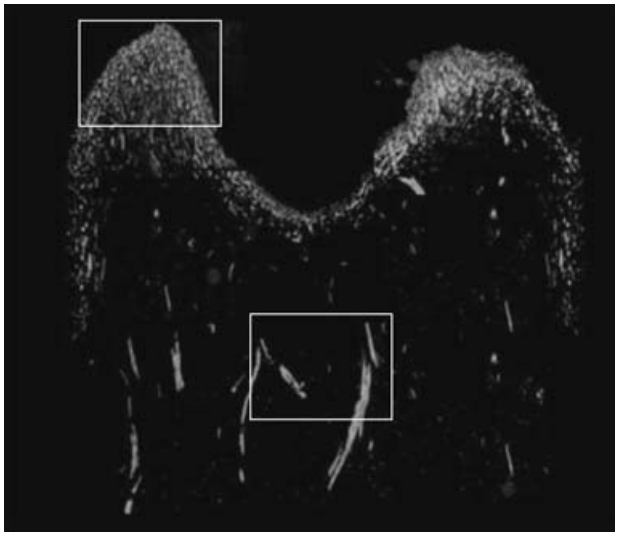

Fig. 1. Composite photomicrograph of the overall innervation of the coronal tooth pulp showing the two different areas employed for quantitative analysis: tip of the pulp horn and mid-coronal region. Each area of analysis represents 0.22 mm^2 of pulp tissue.

from the microscopic image. The percentage area of staining (PAS) for PGP 9.5-, LCA-, and UEIL-labelled tissue was then automatically determined within each field of analysis.

Root measurement

In order to determine the degree of root resorption for each extracted tooth, an established protocol was followed as described by previous authors[9,16]. Firstly, the distance between the enamel–cement junction (CEJ) and the deepest point of root resorption was measured by one investigator (J.M.) using an electronic millimetre calliper (Digimatic Caliper, Mitutoyo, U.K. Ltd, Halifax, West Yorkshire, UK). For each molar, the most resorbed root was selected for this purpose. The resultant measurement was then divided by the expected pre-resorption total root length for that specific tooth type according to Kramer and Ireland's published norms[22]. The overall percentage of root resorption was then calculated for each sample as follows:

$$\%\ \text{root resorption} = \frac{100 - \text{distance from CEJ to point of greatest resorption (in mm)} \times 100}{\text{expected pre-resorption root length (Kramer and Ireland's norms)}}$$

After determining the percentage root resorption by the method described, teeth were subdivided into three groups: (i) group 1: teeth with less than one-third root resorption; (ii) group 2: teeth with one-third to two-thirds root resorption; and (iii) group 3: teeth with more than two-thirds root resorption.

Root length was measured blind to the patient's age and histological findings. A repeat measurement was taken for 20% of the sample 2 months after the initial measurement. A Bland–Altman plot was then generated to estimate intra-examiner agreement[23].

Statistical analysis

One-way analysis of variance (ANOVA) was employed to test for statistically significant differences for mean PAS PGP 9.5, LCA, and UEIL according to the degree of root resorption (< 1/3, 1/3–2/3, > 2/3 resorption). Significance levels were set at $P < 0.05$. Statistical analysis was performed on logarithmically transformed data, but the data are presented graphically in their raw form.

Results

Study sample

A total of 64 upper and lower primary molars from 33 patients were subject to analysis. The mean age of the children was 6.3 years (range 3.0–10.9; SD = 1.8). Thirty-one teeth had less than one-third of their root resorbed (group 1), 25 teeth demonstrated between one-third and two-thirds root resorption (group 2), and only eight teeth had resorption of greater than two-thirds of their original root length (group 3). Very good intra-examiner agreement was found for root length measurement, with all measurements being within the 95% limits of agreement. The mean difference between the initial and repeat measurements was 0.15 mm, with a confidence interval ranging from –0.61 mm to +0.31 mm.

Innervation

Labelling for PGP 9.5 provided excellent visualization of the overall pulpal innervation, and findings were similar to those reported in previous anatomical studies[21]. The pulp horn was the most densely innervated region, with multiple free-ending fibres extending towards the pulp–dentine junction. A well-defined sub-odontoblastic plexus was observed around the pulp periphery, and small/medium-sized nerve trunks (often in association with a blood vessel) were present within the mid-coronal region. A marked variation in innervation pattern, however, was observed in the pulp horn region of some samples with advanced root resorption. In some specimens, there was a reduction in overall innervation density, whereas other samples presented with a very dense, varicose but fragmented innervation (Fig. 2). Figure 3a shows pooled data for mean PAS for PGP 9.5 (innervation density) within the three root resorption subgroups. Interestingly, statistical analysis confirmed that there

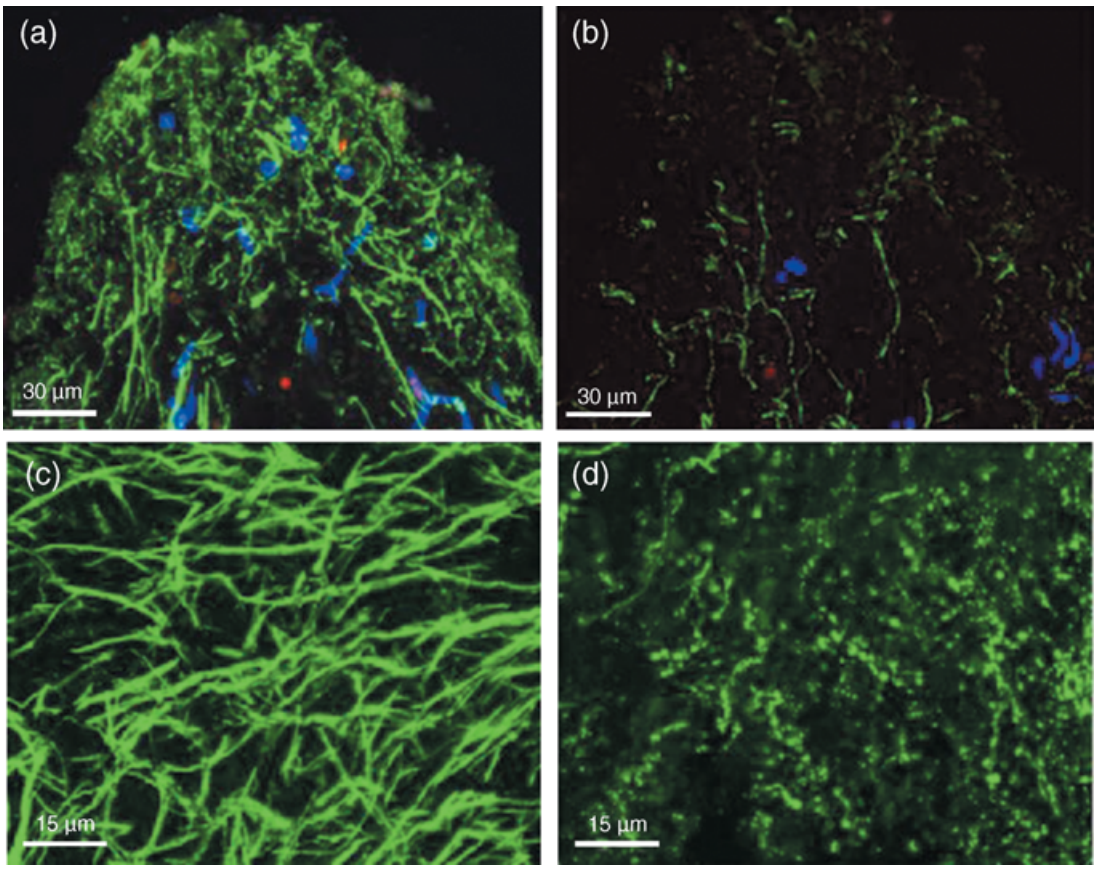

Fig. 2. Digital photomicrographs showing pulps labelled for protein gene product 9.5 (PGP 9.5) (green), leucocyte common antigen (LCA) (red), and *Ulex europaeus* agglutinin I lectin (UEIL) (blue), to demonstrate qualitative differences in the distribution and morphology of PGP 9.5-labelled nerves (green) in the pulp horn and mid-coronal region of primary teeth at different stages of root resorption. (a) Pulp horn region of a tooth with < 1/3 root resorption, showing a normal, well-defined innervation. (b) Pulp horn region of a tooth with > 2/3 root resorption showing a more sparse innervation. (c) Mid-coronal pulp of a tooth with > 2/3 resorption showing a dense innervation. (d) Mid-coronal pulp of a tooth also with advanced root resorption but with varicose and fragmented nerve fibres.

were no significant differences in overall innervation density according to the degree of root resorption in either the pulp horn or mid-coronal region ($P = 0.87$ and $P = 0.15$, respectively, ANOVA).

Immune cells

Round LCA-positive cells were seen to be scattered throughout the coronal pulp tissue of all samples. Generally, findings were similar to those previously reported for non-carious, non-resorbed primary teeth[24]. Dense clusters of immune cells, however, were occasionally observed in both the pulp horn and mid-coronal region of some samples with advanced root resorption (Fig. 4).

It can be seen in Fig. 3b that mean PAS for LCA appeared greatest in teeth with one-third to two-thirds root resorption in both the pulp horn and the mid-coronal region. These were not, however, statistically significant differences ($P = 0.64$ and $P = 0.86$, respectively, ANOVA).

Vascularity

The overall distribution of blood vessels was similar to that described in previous reports with numerous capillaries aligned around the pulp periphery and larger vessels (arterioles and venules) within the mid-coronal region[25]. Quite profound changes in vascular status, however, were noted in some teeth with more than one-third root resorption. There appeared

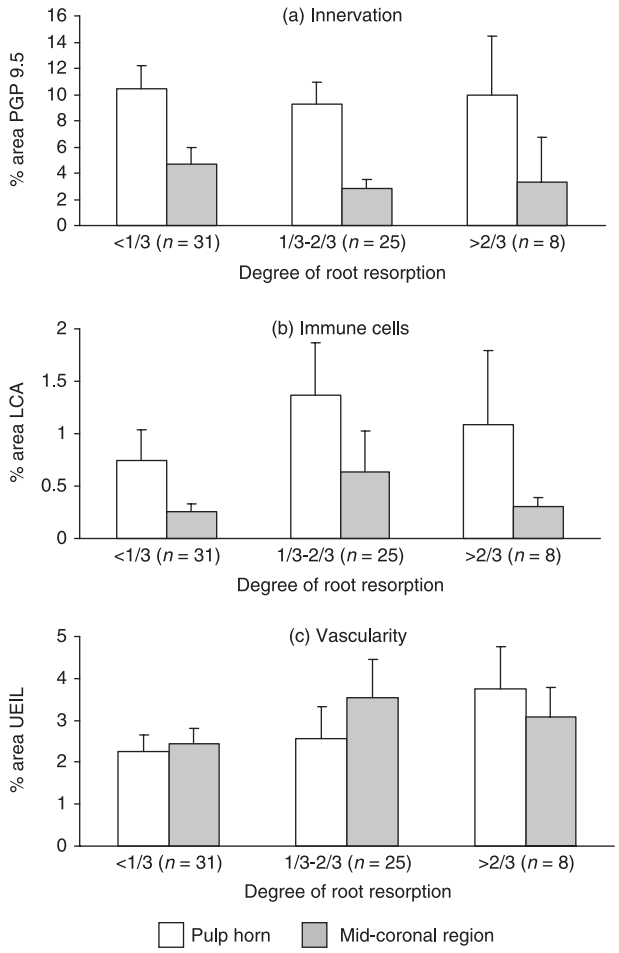

Fig. 3. Bar charts showing the mean (± SEM) percentage area of (a) protein gene product 9.5 (PGP 9.5) labelled tissue (b) leucocyte common antigen (LCA) labelled tissue, and (c) *Ulex europaeus* I lectin (UEIL)-labelled tissue within the pulp horn and mid-coronal region of pulp according to the degree of root resorption.

to be both an increase in large and small vessel dimensions, as well as an increase in capillary number (Fig. 5).

Figure 3c shows pooled data for mean UEIL within the two areas of analysis. Although there was a trend for increased vascularity in samples with more than one-third root resorption, this was not found to be statistically significant ($P = 0.30$ and $P = 0.79$, respectively, ANOVA).

Discussion

This immunocytochemical study has shown that there are no statistically significant differences in overall mean pulpal innervation density, immune cell accumulation, or vascularity in human primary teeth according to the degree of root resorption. Unfortunately, there are no previous quantitative data for innervation density or vascularity with which our findings may be compared. Data have been published, however, for immune cell accumulation with several authors reporting a significant increase in immune cell number with advanced root resorption[10,11,15]. One explanation for this apparently conflicting finding is the difference in methodological approach taken by various investigators: variation in sampling techniques, visualization of immune cells, and quantification may all have a considerable effect on outcomes. Furthermore, with one exception[16], previous studies have employed carious teeth as the experimental material. There is indisputable evidence that there are significant increases in immune cell accumulation within the pulps of carious primary teeth with no physiological root resorption[24]. Thus, it cannot be concluded that the resorptive process alone, in the absence of any other tissue insult, is associated with a significant inflammatory response.

It is interesting, however, that a small number of teeth in this study did have a dense immune cell accumulation. These pulps tended to be from teeth with advanced root resorption in children aged 9–10 years. The more marked inflammatory response, seen in these specimens, may have coincided with a period of increased resorptive activity: it is well recognized that the resorption process includes periods of activity as well as quiescence[11,26]. It is also speculated that teeth in older children may have greater tooth tissue loss, from erosion and/or attrition, thus facilitating ingress of oral bacteria and resultant pulpal inflammation. Teeth with extensive tooth tissue loss and exposed dentine were excluded from the study sample, but it is possible that primary molars in the older children did have a reduced enamel thickness than teeth from younger children.

The tooth pulp is one of the most densely innervated of all human tissues, receiving a predominantly sensory (nociceptive) innervation. Traditionally, the literature has considered the functionality of this dense innervation in relation to pain perception. Current thinking, however, places pain perception as a secondary

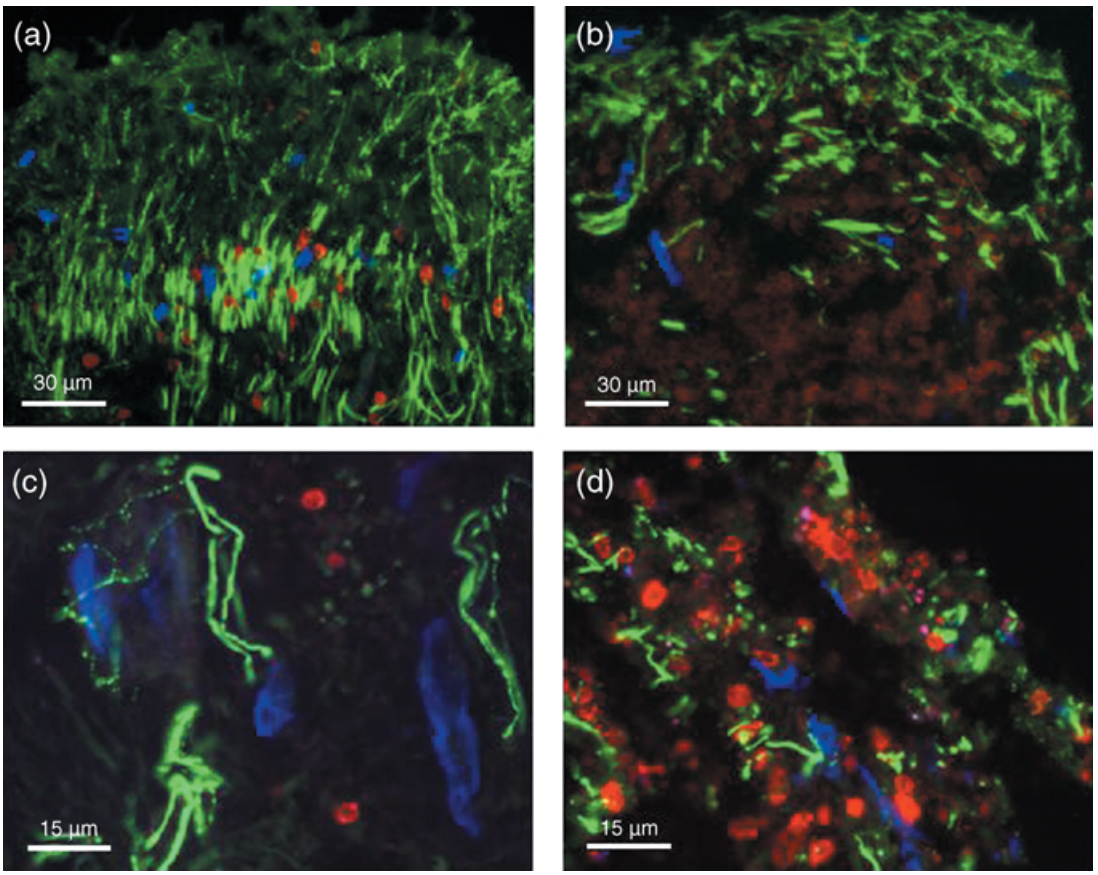

Fig. 4. Digital photomicrographs showing pulps labelled for protein gene product 9.5 (PGP 9.5) (green), leucocyte common antigen (LCA) (red), and *Ulex europaeus* agglutinin I lectin (UEIL) (blue), to demonstrate qualitative differences in the distribution and morphology of LCA-labelled immune cells (red) in the pulp horn and mid-coronal region of primary teeth at different stages of root resorption. (a) Pulp horn region of a tooth with < 1/3 root resorption, showing a moderate number of immune cells. (b) Pulp horn region of a tooth with > 2/3 root resorption showing a dense accumulation of immune cells. (c) Mid-coronal pulp of a tooth with 1/3–2/3 root resorption showing minimal scattered immune cells. (d) Mid-coronal pulp area of a tooth with > 2/3 root resorption showing a dense accumulation of immune cells.

function, with intradental nerves playing a more important role in defence and healing[19]. Experimental studies in animal models have shown that a favourable response to tooth injury is dependant on an intact sensory innervation[27]. Thus, the innervation status of resorbing primary teeth has clinical relevance, not only in relation to pain processing, but also in terms of the potential to heal and repair following injury such as caries or restorative interventions.

This study did not identify any appreciable decreases in mean innervation density in teeth with root resorption, thus it would appear that they retain the ability to mount a defence response following an insult. This, however, is a tentative proposal as anatomical findings may not directly equate to function. Supporting studies would be necessary to establish the physiological responses of pulpal nerves to various stimuli. Furthermore, it is acknowledged that individual tooth samples did demonstrate abnormal nerve morphology. Within the pulp horn region, nerve terminals were seen to demonstrate a more beaded and fragmented appearance with some decrease in overall innervation density. These findings may be suggestive of neural degeneration as similar observations have been made in the adult ageing dentition where nerve fibre varicosities, vacuolations, fragmentation, and a sparser innervation have been reported[17]. The reason for the wide inter-sample variation in innervation status seen in teeth with similar degrees of root resorption is

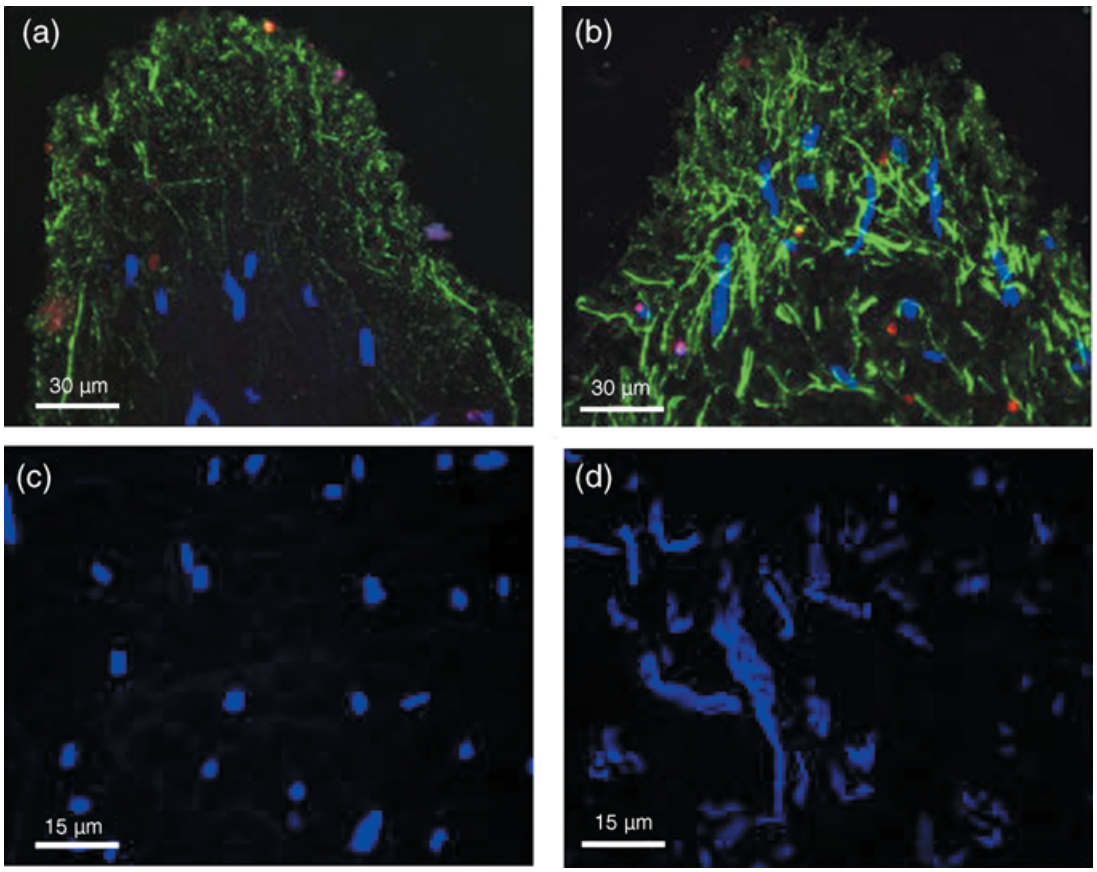

Fig. 5. Digital photomicrographs showing pulps labelled for protein gene product 9.5 (PGP 9.5) (green), leucocyte common antigen (LCA) (red), and *Ulex europaeus* agglutinin I lectin (UEIL) (blue), to demonstrate qualitative differences in the distribution and morphology of UEIL-labelled blood vessels (blue) in the pulp horn and mid-coronal region of primary teeth at different stages of root resorption. (a) Pulp horn region of a tooth with < 1/3 root resorption showing normal distribution of blood vessels. (b) Pulp horn region of a tooth with > 2/3 root resorption and a small increase in blood vessel number. (c) Mid-coronal pulp region of a tooth with < 1/3 root resorption showing normal vascularity. (d) Mid-coronal pulp region of a tooth with > 2/3 root resorption showing enlarged blood vessels.

not clear and warrants further investigation. One explanation may be that the categorization of root resorption in this study (< 1/3, 1/3–2/3, > 1/3) was too broad, thus possible correlations between the exact percentage of root resorption and pulpal status were masked.

General dental practitioners are sometimes reluctant to give local anaesthetic for restorative procedures in primary teeth for a variety of reasons, including the commonly held belief that resorbing teeth are less sensitive to pain. On the basis of this anatomical study, there is no conclusive evidence for a universal degeneration of intrapulpal nerves with advanced physiological root resorption. Thus, in the absence of any contrary functional evidence, the need for appropriate analgesia when restoring resorbing primary teeth would seem to be upheld.

Although not statistically proven, this study identified a trend for an increased vascularity with progressive root resorption, particularly within the pulp horn region. This may reflect the higher metabolic demands of odontoblastic cells during active phases of resorption. The presence of a good blood supply may have considerable biological importance in provision of nutrients and removal of metabolic waste products. Alternatively, as previous investigators have suggested, this finding may be a reflex of the resorption process itself, as a consequence of the widening of the apical area[9]. Enlarged blood vessels, some with a lymphatic morphology, were observed in late stages of root resorption

in some samples. These findings concur with those of Bolan and Rocha who reported hyperaemia and dilated blood vessels in their qualitative study of resorbing primary teeth[14].

It is important to reflect on accepted clinical practice for the management of the carious primary dentition in the light of these findings. The presence of a 'normal' or minimally inflamed tooth pulp is considered a prerequisite for the success of procedures such as indirect pulp capping or vital pulptomy[28,29]. As this study has found that (non-carious) teeth with advanced root resorption retain their normal pulpal status, these procedures would not appear to be contraindicated. It would, however, be necessary to develop this research further to actually compare caries-induced pulpal responses in non-resorbed versus resorbing primary teeth. Furthermore, an acknowledged limitation of this study was the comparatively small number of samples in the greater than two-thirds root resorption subgroup compared to the other two groups, and future research should aim to include more teeth in the advanced stages of resorption.

Finally, it is interesting to note that The British Society of Paediatric Dentistry clinical guidelines for pulp treatment of the primary dentition cite that pulp therapy is contraindicated in teeth with more than two-thirds root resorption[30]. Although the rationale for this statement is not given, it may be inferred that extensive treatment is not indicated for a tooth close to exfoliation, due to its limited life expectancy within the mouth, rather than concerns about the tooth's biological potential for healing and repair.

Conclusion

This is the first study to quantify changes in pulpal innervation density, immune cell accumulation, and vascularity in caries-free teeth during physiological resorption. Although considerable intersample variation was observed, there were no statistically significant mean changes in these variables overall. Within the obvious limitations of a purely anatomical study, it would appear that resorbing teeth do retain the structures necessary for pain perception, healing, and repair.

What this paper adds

- This study has provided a comprehensive insight into the effect of physiological root resorption on pulpal status.
- It has shown that mean pulpal innervation, immune cell accumulation, and vascularity remain remarkably unaffected during tooth exfoliation.

Why this paper is important to paediatric dentists

- Paediatric dentists routinely provide pulp therapies for primary teeth at different stages of dental development. It is important that clinicians have an understanding of pulp biology and how this may affect their treatment decisions

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