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Type 2 diabetes and the clinically normal pulp: An *in vitro* study

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Abstract

Aim: The aim of this study was to investigate the effect of type 2 diabetes (T2D) on clinically normal dental pulp tissue by using special stains and immunohistochemistry (IHC) to determine the morphology of the coronal pulp and distribution of immune markers in non-T2D and T2D groups.

Methodology: Ethics approval for this *in vitro* pilot study was obtained from the University of Otago Human Ethics Committee (16/069). Twenty extracted permanent molar teeth diagnosed as having clinically normal pulp status were collected. Ten teeth were from participants with well-controlled T2D and ten from participants without diabetes (non-T2D). Each tooth was sectioned transversely at the cemento-enamel junction before the crowns were decalcified and embedded in paraffin. Sections were stained with haematoxylin and eosin, Massons trichrome, and van Gieson stains for histological and morphological evaluation. IHC using anti-CD4, anti-CD68 and anti-CD83 and anti-IL1β, anti-IL6, anti-IL17, anti-TNF-α, anti-TLR2, anti-TLR4 and anti-FOXP3 identified proteins of interest. Qualitative and semi-quantitative analyses evaluated the morphology of the dental pulp and protein expression. Data analyses were performed with GraphPad Prism, using Student's *t*-test and multiple regression using SPSS at $p < .05$.

Results: Special stains demonstrated morphological differences in the T2D dental pulp compared with non-T2D. Qualitative analysis indicated that the pulp in the T2D samples was consistently less cellular, less vascular, showed evidence of thickened blood vessel walls, increased pulp calcification and collagen deposition. Semi-quantitative analysis of IHC samples showed the T2D pulp had significantly increased expression of macrophage and dendritic cell markers CD68 ($p < .001$) and CD83 ($p = .04$), and there was significantly greater expression of inflammatory cytokines IL1β ($p = .01$), IL6 ($p < .0001$), IL17 ($p < .0001$) and TNF-α ($p = .01$). T2D samples showed a significant increase in markers of innate inflammation, TLR2 ($p < .001$) and TLR4 ($p < .001$) and decreased expression of regulatory T-cell marker, FOXP3 ($p = .01$). Multiple regression showed that age-corrected differences were statistically significant.

Conclusion: Preliminary findings suggest that T2D may exert a similar response in the pulp to complications in other body sites. Hyperglycaemia is associated with changes in the morphology of the clinically normal dental pulp with altered immune cell and cytokine expression.

KEYWORDS
dental pulp, histomorphology, immune response, immunohistochemistry, type 2 diabetes

INTRODUCTION

Type 2 diabetes (T2D) is a chronic multisystem metabolic disorder characterized by hyperglycaemia, which effects the structure, function and the immune response associated with many organs and tissues. Elsewhere in the body, T2D commonly presents with complications of fibrosis, retinopathy, neuropathy and cardiovascular disease, and these have multifactorial causes (Daryabor et al., 2020). T2D is a pro-inflammatory condition (Al-Shukaili et al., 2013; Tsalamandris et al., 2019) and peripheral vascular changes in patients with the disease are associated with altered immunological responses, increased TNFα, IL6, IL1β and IL17 (Berbudi et al., 2020; Dandona et al., 2007; Hang et al., 2014). Furthermore, T2D is associated with decreased immunosuppressive regulatory T-cell (Treg) activity and increased pro-inflammatory cytokines posing a greater risk of infection (Qiao et al., 2016; Samuel et al., 2019). How T2D influences the pulp immunity is unclear.

Toll-like receptors (TLR) are pattern recognition receptors, which play a central role in the innate immune system. Within the pulp, TLR2 and TLR4 are expressed in healthy and inflamed human dental pulp, particularly on odontoblasts where they form the first line of defence to irritants by acting as antigen-presenting cells (APC) via the innate immune response (Farges et al., 2009; Mutoh et al., 2007). In the presence of T2D, the function of other APCs, including dendritic cells, monocytes and neutrophils cells, is reduced (Daryabor et al., 2020). Tregs are a specialized population of T cells, which act to suppress the immune response and inhibit cytokine production within tissues. In patients with T2D who also have periodontal disease, these cells are reduced but it is unknown how T2D influences the expression of TLRs and Tregs within the dental pulp (Gaudin et al., 2015; Qiao et al., 2016).

There is abundant evidence that T2D is associated with pathological changes in the oral tissues (Kudiyirickal & Pappachan, 2015). This is particularly so in the periodontal tissues where it induces tissue destruction from decreased collagen turnover, thickening of the walls of the gingival blood vessels and narrowing of the vessel lumen, and inducing vascular dysfunction (Preshaw & Bissett, 2013). In other body sites, T2D also results in changes to collagen within the tissue and the development of atherosclerosis, thickening of capillary basement membranes, increased arterial wall stiffness, decreased tissue elasticity, sclerosis of the renal glomeruli, stiffening of the heart muscle, periarticular rigidity and osteoarthritis (Poznyak et al., 2020; Rask-Madsen & King, 2013; Veronese et al., 2019).

Understanding the effects of T2D on the morphology of the normal dental pulp and its clinical implications in pulp and periapical pathology are still evolving with most knowledge arising from *in vitro* histological studies and clinical cases (Garber et al., 2009; Lima et al., 2013; Rudranaik et al., 2016). Rats induced with hyperglycaemia show a marked reduction in plasma blood flow within the dental pulp with an increased amount of fibrous connective tissue and arteriosclerotic change in the walls of blood vessels (Amatyakul et al., 2003; Garber et al., 2009). This can affect dental pulp repair and healing as evidenced by reduced dentine bridge thickness following direct pulp capping procedures with mineral trioxide aggregate (MTA; Garber et al., 2009). Furthermore, scanning electron microscopy studies have demonstrated physicochemical differences in dentine from patients with diabetes, which can negatively influence clinical outcomes. The shear bond strength of composite resin to dentine and enamel is reduced in diabetes and within root dentine there is significantly increased dentinal tubular density and diameter (Saghiri et al., 2020, 2021). Together, these increase the potential for persistent infection from microbial leakage and dentinal tubule invasion.

There is increasing evidence of an association between the presence of local pulp and periapical inflammation resulting in apical periodontitis, the outcome of root canal treatment, tooth loss and T2D (Cabanillas-Balsera et al., 2019; Gupta et al., 2020; Nagendrababu et al., 2020; Pérez-Losada et al., 2020; Segura-Egea et al., 2016, 2019). There is a greater prevalence of periapical lesions in patients with T2D compared with non-T2D patients. Patients with T2D have a greater likelihood of persistent periapical disease following endodontic treatment and significantly more root filled teeth are extracted in patients with systemic disease. Despite this association, the likely presence of confounding variables means it is not possible to define a causal relationship

(Segura-Egea et al., 2019). Although T2D can therefore be considered a prognostic factor in the management of advanced pulp disease and endodontic outcomes, what is less clear is how the coronal pulp is influenced by hyperglycaemia.

Clinically, determining the pulp health status is based on subjective and objective clinical findings. Treatment outcomes are variable, and it can be even more challenging for clinicians to diagnose the pulp status needed to guide treatment decisions and tooth prognosis for patients who have associated systemic disease. The recommendations for diagnosing the inflammatory state are the history, clinical examination, pulp sensibility testing (electric and cold) and viewing diagnostically acceptable periapical radiographs (Duncan et al., 2019). The term 'clinically normal pulp' is used to describe an asymptomatic, uninflamed dental pulp that clinically and radiographically has no signs or symptoms of disease (American Association of Endodontists, 2009). Cells within healthy (normal) tissues are necessary for immune surveillance and contribute to healing, but our understanding of the 'normal' morphological appearance of the pulp in a patient with T2D is unclear and warrants investigation. Furthermore, an inflammatory response is necessary to initiate healing and there is sparse understanding around the effect of hyperglycaemia on common immune and inflammatory markers within the 'normal' dental pulp. No other *in vitro* studies were identified in the literature which examined the human dental pulp using histology and immunohistochemistry (IHC) techniques and the ease of tissue preparation for T2D tissue samples was unknown, so a pilot study was justified. Therefore, the aim of this *in vitro* study was to investigate and provide preliminary findings on the effect of T2D on the morphology of the dental pulp and the expression of proteins associated with the immune system. The hypothesis was that T2D will result in altered morphology within the clinically normal pulp with increased connective tissue and the proteins associated with the immune system and the pulp will be affected similar to other body sites.

METHODS AND MATERIALS

Ethics approval for this study was obtained from the University of Otago Human Ethics Committee (Health; Ref. H16/069). This was a pilot study, and patients who were having mature human permanent molar teeth extracted for clinical reasons as part of a treatment plan, and who met the inclusion criteria were selected. Teeth were collected from T2D ($n = 10$) and non-T2D patients ($n = 10$). Prior to extraction, teeth were diagnosed clinically as having a 'normal pulp' using established clinical criteria (American Association of Endodontists, 2009; Duncan et al., 2019). Teeth were non-carious and unrestored, asymptomatic and responded within normal limits to sensibility testing. They were not sensitive to percussion or palpation tests and radiographically had a normal periapical appearance.

Inclusion criteria were patients aged 30–55 years who required extraction of a molar tooth with a 'normal' pulp for clinical reasons eg as part of a dental clearance of third molars. Participants were either non-diabetic (normal HbA1c (39–45 mmol/mol or 5.7–6.4%) or well-controlled T2D (diagnosed by a physician based on medical records, lifestyle factors and successive HbA1c laboratory tests (50–55 mmol/ml or 6.7–7.2%). Only patients who had been diagnosed with T2D for more than 12 months had their diabetes controlled and were free from other systemic disease were included in the study. Participants were excluded if they were smokers, pregnant, had taken antibiotics within the previous 3 months, were on regular anti-inflammatory medication or had pulpitis, periodontitis or evidence of tooth wear.

Sample preparation

Each tooth was sectioned horizontally below the cemento-enamel junction, and the coronal portion immediately placed in 10% neutral buffered formalin for 24 h. The specimens were decalcified in 10% ethylenediaminetetraacetic acid solution (EDTA; pH 7.4), trimmed and placed in phosphate-buffered saline for 24 h to remove residual EDTA. Each tooth was formalin fixed and embedded in paraffin and tissue sections, 4 μm thick, were obtained.

Histological staining

The first section was stained with haematoxylin and eosin (H&E) for histological examination by two oral pathologists blinded to the sample selection to confirm the relative absence of inflammation. Additional sections were stained with Masson's trichrome and van Gieson (VG) special stains to identify the presence and location of collagen, connective tissues and blood vessels within the samples. The distribution of staining was compared between the T2D and non-T2D groups. Samples were analysed visually with the non-T2D group used as the baseline for comparison. Positive control tissues were run in parallel, kidney for Masson's trichrome and intestine for VG stain.

Immunohistochemistry

Monoclonal primary antibodies were used to examine protein expression in the tissue samples: anti-CD4 (1.41 μg/ml, AbCam), anti-FoxP3 (20 μg/ml, Santa Cruz Biotechnologies), anti-TLR2 (10 μg/ml, Santa Cruz Biotechnologies), anti-TLR4 (10 μg/ml, AbCam), anti-CD68 (0.05 μg/ml, AbCam), anti-IL17 (2 μg/ml, AbCam), anti-CD83 (4 μg/ml, AbCam), anti-IL-6 (10 μg/ml, AbCam), anti-TNFα (1 μG/ml, AbCam) and IL-1β (10 μg/ml, AbCam). A heat-induced antigen retrieval technique was used to unmask tissue antigens. A preheated 0.01 M sodium citrate buffer (pH 6.0) at 80℃ for 20 min was used with the exception of anti-IL17, which was heated for 15 min only. The primary antibodies were incubated overnight at 4°C. For isotype-matched control sections, immunoglobulin (Ig)-G replaced the primary antibody at the same concentration. The positive control tissues included lymph node for anti-CD4, anti-FOXP3, anti-TLR2, anti-TLR4, dentigerous cyst for anti-CD68 and anti-IL17, spleen for anti-CD83, anti-IL6 and anti-TNFα, and thymus for anti-IL1β. The binding of the antigen-antibody signal was amplified manually using secondary reagent EnVision™+ Dual Link System-HRP for both the anti-mouse and anti-rabbit (Cat. No. K4063, Agilent Dako). Following signal amplification, the enzyme-labelled antibodies were visualized using 3,3′-diamino-benzidine (Sigma Aldrich) as the chromogen. Finally, the sections were counterstained with Gill's haematoxylin and resin mounted. All samples were assigned a random numerical specimen number, which enabled examiners to be blinded during data analysis.

Tissue morphology

All sections were examined using light microscopy with an overall morphological survey to observe the morphology of blood vessels, odontoblasts, cells, nerve bundles and extracellular matrix (ECM) components of the pulp. Fibrosis was defined as an increased collagen fibre concentration and was noted as present or absent. The presence of pulp calcifications was also noted. The dentine and predentine were observed and described for both groups.

Semi-quantitative evaluation of cells and blood vessels using H&E and special stains

Cell nuclei, free in the pulp connective tissue, and blood vessels were counted in the pulp and results were validated by a second examiner blinded to the experiment. Blood vessels were defined as endothelial cells lining a vessel lumen and the density of blood vessels (number per field area) was determined. For each sample, five defined areas in the centre of the pulp with the greatest number of cells were selected for analysis. Cell counting was performed manually at 40× magnification, and the mean number of cells and blood vessels per unit area was determined for each sample.

Analysis of immunohistochemistry staining

Semi-quantitative analysis of expression of proteins was performed by randomly selecting ten areas with high cell density within the pulp using ImageScope (Aperio ImageScope version 12.4.3; Aperio Technologies, Leica Biosystems). The size of each area was defined as that corresponding to the viewing field of 0.028 mm^2 (40× magnification). The results were expressed as a mean of the positive cells per unit area for each antibody.

Statistical analysis

Quantitative analysis was performed using GraphPad Prism 7 for Mac OS X (GraphPad Prism 7.0a Macintosh Version, 2016). Student's *t*-test was used to compare differences between the non-T2D and T2D dental pulp samples. A *p*-value of $<.05$ was considered statistically significant. A multiple regression analysis was carried out to investigate whether age influenced the assessments in the quantitative analysis of markers for T2D and non-T2D samples.

RESULTS

The mean age of non-T2D participants was 46 years ±4.5 standard deviation (SD) and 50 years ±4.9 SD for T2D, and there was no statistical difference in age between the groups ($p = .07$). There were five males and five females in each group and the mean time since diagnosis was 2.4 years. The mean HbA1c of the T2D patients was 52.7 mmol/mol ±1.5 SD or 7% ±0.2 SD. Gross evaluation of the pulp from non-T2D participants showed that it was pink, soft in consistency and resilient, whereas pulp tissue from the T2D participants was harder in consistency and friable. T2D specimens consistently required an additional 2 weeks for hard tissue decalcification, and the friable nature of the tissue required very careful handling during cutting of the specimens and mounting on histological slides.

Histology and morphology

The general histology of the pulp was observed on H&E-stained sections and were found to have a consistent appearance for all samples in each group with no differences were observed between male and females. All T2D samples showed less definition between the cell-free and cell-rich zones, and the tissue appeared to be less cellular with fewer cells and blood vessels compared with non-T2D samples. Furthermore, T2D samples showed obvious changes in the predentine region and calcifications (Figure 1a,b).

The pulp in both groups consisted of fibrous connective tissue surrounded by tubular dentine and a layer of unmineralized predentine lined by odontoblasts (Figure 1c). However, in T2D samples, the odontoblast layer was less well defined, and discontinuous in some regions, with clear changes in the position and orientation of the odontoblast nuclei (Figure 1d). Odontoblasts were more cuboidal compared with those in the non-T2D dental pulp and the junction between the odontoblasts and predentine was irregular with changes in thickness along the odontoblast layer (Figure 1d). Irregular tertiary dentine-like tissue and numerous pulpal calcifications were observed in eight of the ten T2D samples (Figure 1b,d,g,h). Mineralized deposits of various sizes were seen within the coronal pulp chamber and were commonly observed free in the soft tissue or close to blood vessels (Figure 1g,h). Within the T2D pulp, most blood vessels appeared to have thickened and irregular walls with fewer endothelial cells (Figure 1f).

Masson's trichrome and VG stains confirmed the H&E observation of denser collagen in the central region of the T2D dental pulp compared with non-T2D samples and thicker walls of the blood vessels in the central pulp of T2D samples (Figure 2). Furthermore, the VG stain identified endothelial cells lining blood vessels and the nerve fibres and neurovascular bundles were less distinct or degenerate in most T2D samples.

The T2D samples had significantly less cell nuclei ($p < .001$) free within the pulp connective tissue, and samples were less vascular ($p = .03$) than non-T2D samples. The mean number of cells within the ECM of the T2D pulp was 776.60 ± 42.44 SD, compared with 1005 ± 31.66 SD in the non-T2D pulp. In the T2D pulp, the mean density of blood vessels was 180.8 ± 4.85 SD compared with 197.8 ± 5.75 SD in the non-T2D pulp (Figure 3).

Immunohistochemistry

Overall, there was increased expression of immune cells, particularly dendritic cells and macrophages, and cytokines throughout the T2D pulp samples compared with samples in the control group. There were more significantly more CD68+, CD83+, IL1β, IL6, IL17 and TNF-α in T2D samples (Table 1). Similarly, TLRs which are important in the innate immune response were also upregulated with significantly increased expression of TLR2 and TLR4+ cells present in the T2D pulp (Table 1). A multiple regression analysis was conducted, and overall assumptions of linearity, normally distributed errors and collinearity were checked and met (Table 2). The correlation of the relationship between the expression of inflammatory markers with diabetes status and age showed that whilst the diabetes status contributed significantly to the outcome, the 5-year difference in mean participant age did not influence assessments (Table 3). Qualitative analysis showed that the protein expression for TLR4 was particularly evident in the odontoblast and sub-odontoblast region. In non-T2D samples, Tregs were commonly observed in the region of blood vessels, but these were significantly decreased in the presence of T2D. The validity of the staining protocol was confirmed with immunopositive lymph node and negative monoclonal IgG control samples (Figure 4).

DISCUSSION

The present study suggests T2D can lead to morphological changes in the clinically normal dental pulp with some resemblance to an 'aged' pulp. Furthermore, this work indicates that when corrected for age, there may be changes in the immune system in the pulp as a response to hyperglycaemia, with pro-inflammatory effects observed.

Individual responses and biological variability to a change in glycaemic levels are multifactorial and glycaemic control is the foundation for preventing long-term complications of T2D. In contemporary practice, physicians have moved from gluco-centric model for diabetes control to use a more patient approach which includes an individualized target HbA1c level between 6.7% and 7.2% alongside other diabetes management tools to evaluate 'control' (Rodriguez-Gutierrez et al., 2019). In this study, successive HbA1c tests and lifestyle factors were evaluated; however, it must be acknowledged that changes observed within the pulp are likely to have occurred over a longer time.

A limitation of the current study is the small sample size; however, this was a pilot study intended to provide preliminary findings and the consistent observation amongst the diabetic samples offers new knowledge. Importantly, most research in this area has been completed on animals and the current study expands understanding and provides information directly related to humans. Furthermore, all patients had well-controlled T2D, had been diagnosed for less than

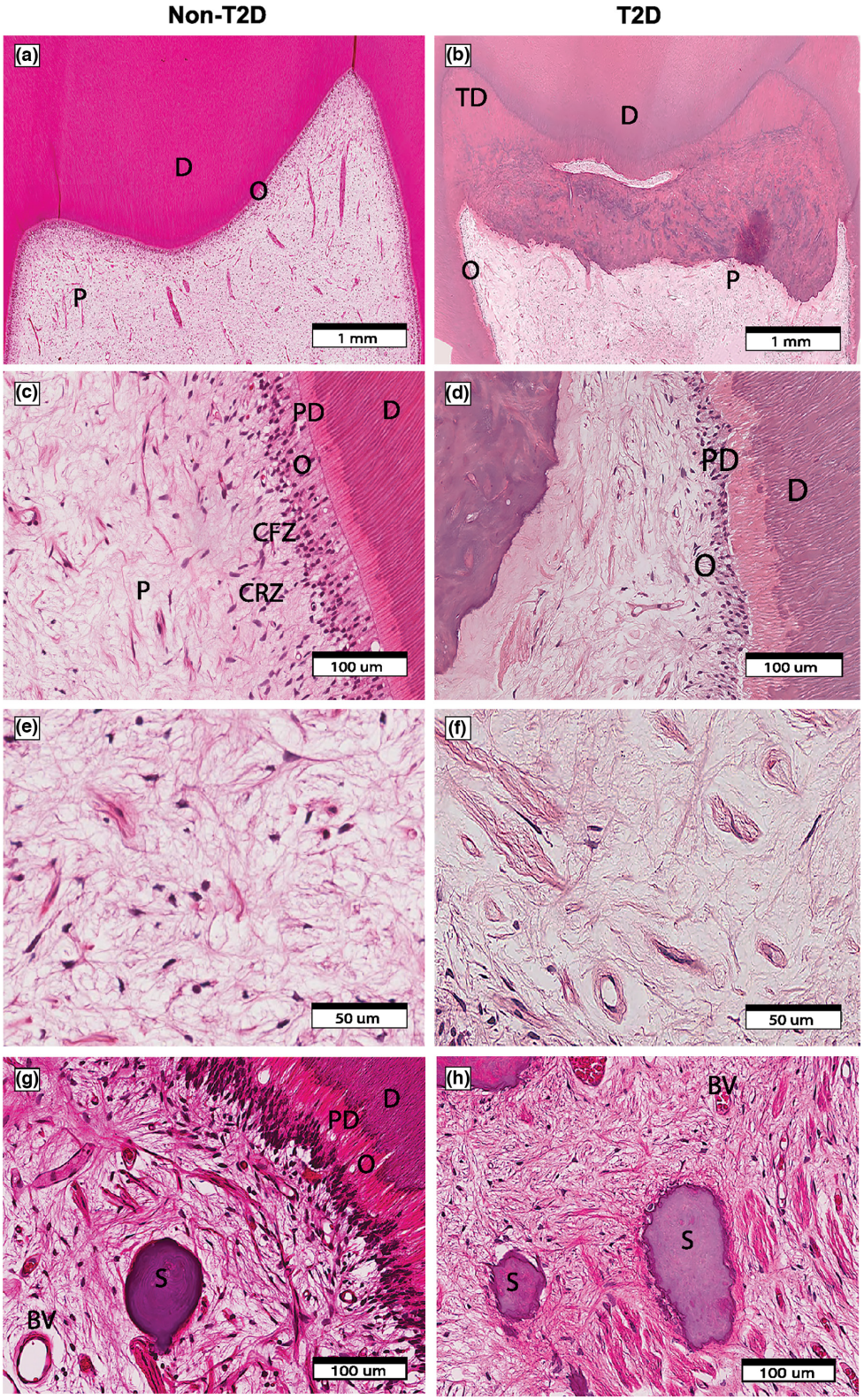

FIGURE 1 Histological images of coronal clinically normal dental pulp from non-T2D and T2D participants (H&E staining); (a), (c), and (e) representative non-T2D dental pulp and (b), (d), (f), (g) and (h) representative TR2D dental pulp. The T2D dental pulp shows denser collagen with a notable presence of tertiary dentine. It appears less cellular, has thickened blood vessel walls, and the presence of amorphous calcified stones within the T2D pulp tissue was common. (a and b; 2× magnification, scale bar, 1 mm, (c),(d), (g) and (h); 20× magnification, 100 μm, e and f; 40× scale bar 50 μm). D, dentine; PD, predentine; O, odontoblast; P, central pulp; CFZ, cell free zone; CRZ, cell rich zone; BV, blood vessels; TD, tertiary dentine; S, pulp calcification/stone

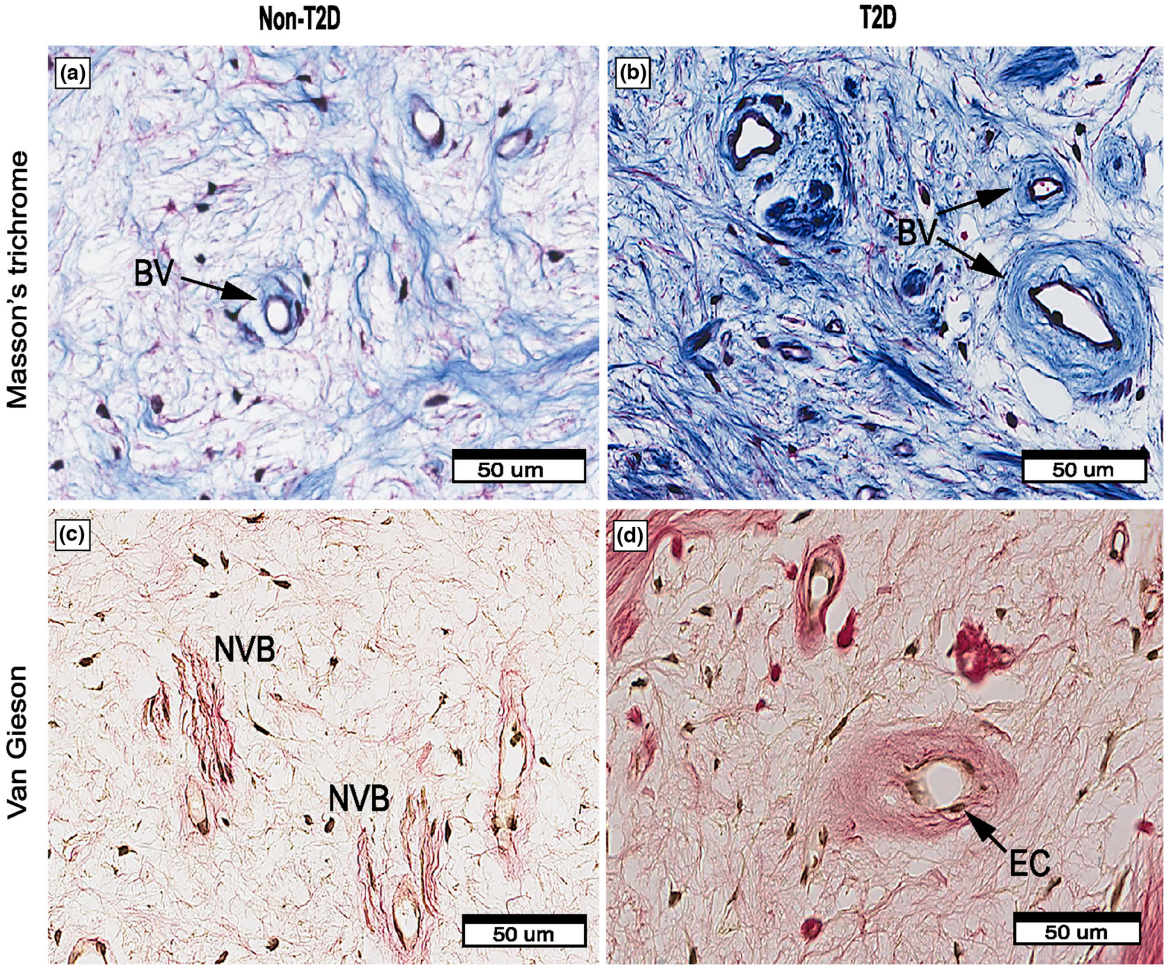

FIGURE 2 Special stain images showing the histomorphology of the clinically normal dental pulp from non-T2D and T2D participants; (a) and (c) representative non-T2D dental pulp and (b) and (d) representative T2D dental pulp; (a) and (b) shows Masson's trichrome stain; the intensity of blue collagen stain in central region of dental pulp and around thickened blood vessels (black arrows) was increased in T2D group. Endothelial cells lining blood vessels are stained black; (c) and (d) shows Van Gieson stain; collagen staining was intense in T2D dental pulp especially around blood vessels compared with the non-T2D dental pulp. The presence of neurovascular bundles was less evident in T2D samples. (a–d; 40× scale bar 50 μm). BV, blood vessels; EC, endothelial cells; NVB, neurovascular bundle

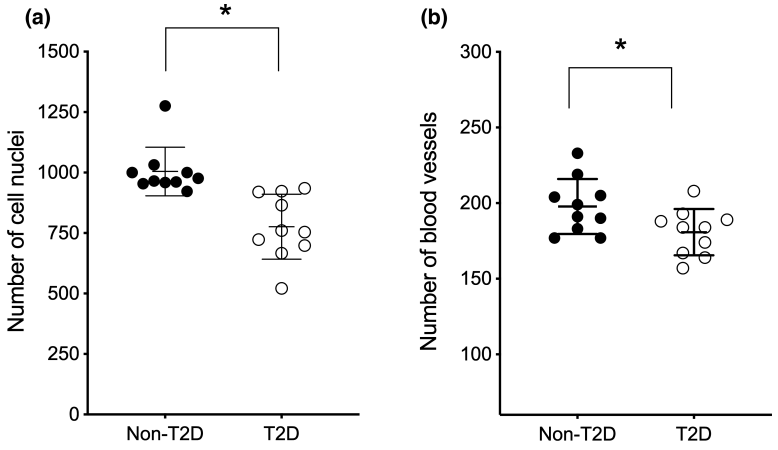

FIGURE 3 Line plot graph showing the mean total non-endothelial cell nuclei and blood vessel count/section in T2D and non-T2D dental pulp samples using an unpaired Student's *t*-test (*p-value .05). The error bar indicates standard deviation

5 years, and although there was a small difference in the mean age of the groups, this was not significant, and findings were carefully checked to exclude changes due to age.

This study showed histological evidence of an increased density of collagen within the connective tissue of T2D samples compared with non-T2D. This is similar

TABLE 1 Mean number and standard deviation (SD) of immunopositive cells for antibody markers of immune cells and cytokines in T2D and non-T2D samples

	Non-T2D		T2D		
Antibodies	**Mean**	**SD**	**Mean**	**SD**	***p*-value**
Anti-CD4	120.40	45.45	137.30	36.17	.37
Anti-CD68	74.80	28.09	144.00	41.74	<.001
Anti-CD83	59.30	31.49	85.40	21.67	.04
Anti-IL1β	80.70	38.55	121.00	23.45	.01
Anti-IL6	139.50	27.79	236.40	42.84	<.0001
Anti-IL17	52.90	17.78	115.20	19.10	<.0001
Anti-TNF-α	97.00	18.69	124.6	24.84	.01
Anti-FOXP3	164.30	42.96	122.50	21.70	.01
Anti-TLR2	28.00	9.17	64.10	21.65	<.001
Anti-TLR4	26.00	13.20	53.30	12.23	<.001

TABLE 2 Summary of multiple regression model analysis for quantitative assessment

Antibodies outcome variable	R^2	**df**	***F* ratio**	***p*-value**
Anti-CD4	.31	2	3.90	.04
Anti-CD68	.58	2	11.66	<.001
Anti-CD83	.56	2	10.65	<.001
Anti-IL1β	.55	2	10.37	.01
Anti-IL6	.92	2	108.0	<.001
Anti-IL17	.80	2	34.31	<.001
Anti-TNF-α	.68	2	7.47	.01
Anti-FOXP3	.41	2	5.80	.01
Anti-TLR2	.77	2	28.6	<.001
Anti-TLR4	.64	2	16.34	<.001

Note: Predictor (constant) T2D status, Age.

TABLE 3 Correlation of the relationship between the expression of inflammatory markers with diabetes status and age

	Diabetes status		Age	
Antibodies	***B* value**	***p*-value**	***B* value**	***p*-value**
Anti-CD4	−22.12	.02*	−1.6	.08
Anti-CD68	−68.93	<.001***	−1.25	.42
Anti-CD83	−28.97	.01**	.22	.77
Anti-IL1β	−43.51	<.001***	−.85	.42
Anti-IL6	−98.24	<.001***	−.36	.63
Anti-IL17	−65.00	.01**	−1.21	.16
Anti-TNF-α	−28.78	.002**	−.31	.71
Anti-FOXP3	42.63	.01**	.22	.88
Anti-TLR2	−36.78	<.001***	−.18	.74
Anti-TLR4	−24.92	<.001***	.63	.27

Note: *$p \leq .05$; **$p \leq .01$; ***$p \leq .001$

to the histological pattern described for cases of chronic pulpitis (Giuroiu et al., 2015) and was consistent with the gross appearance of the pulp in T2D samples, which tended to be fibrous and hard. The localization of type 3 collagen and other connective tissue proteins have been well described in the pulp (Ferreira Martinez et al., 2000); however, the findings from the current study suggest that the presence of hyperglycaemia results in denser connective tissue which may potentially alter the ability of the tissue to heal. A similar increase in connective tissue in TD2 patients has been reported elsewhere in the body (Argyropoulos et al., 2016; Jia et al., 2018). This has been illustrated in the soft tissue of the lungs where T2D is associated with fibrosis from a dysregulated production of the ECM resulting in upregulation of type 3 collagen mediated by TGFβ1 (Talakatta et al., 2018).

Within the pulp, the T2D samples had reduced blood vessel density with thickening of the perivascular collagen. It is known that hyperglycaemia causes changes to vascular walls, which may accelerate arteriosclerosis (Catanzaro et al., 2006). Elevated blood glucose results in reduced elasticity of vessels reducing blood flow, causing damage to small vessels and impeding oxygen and nutrient supply (Rask-Madsen & King, 2013). Together, the pulpal observations in the current study suggest the occurrence of diabetes induced pathological events and indicates that the potential for angiogenesis and healing is likely to be more limited. This includes in instances of deep caries and reversible pulpitis where the balance may be tipped towards disease progression. Furthermore, these findings lend support for reviews and meta-analyses that show T2D is associated with pulp and periapical pathology and should be considered as a prognostic factor in healing following endodontic treatment (Gupta et al., 2020; Segura-Egea et al., 2016, 2019).

The dentine and pulp are intimately related as a complex. T2D has been shown to negatively affect the dentine with clinical implications, and this study has demonstrated morphological changes within the pulp–dentine complex associated with T2D including the presence of irregular tertiary dentine, reduced cellularity and vascularity are like those typically seen in pulps of older adults (Morse, 1991; Saghiri et al., 2020). Our results were validated by closely comparing samples of a similar age and excluded teeth with restorations and occlusal wear. Pulpal calcifications are also more common with increasing age and were consistently observed in diabetic samples. Calcifications have been associated with diabetes elsewhere and described in the dental pulp of rats (Huang & Chen, 2016; Inagaki et al., 2010; Liabeuf et al., 2014;

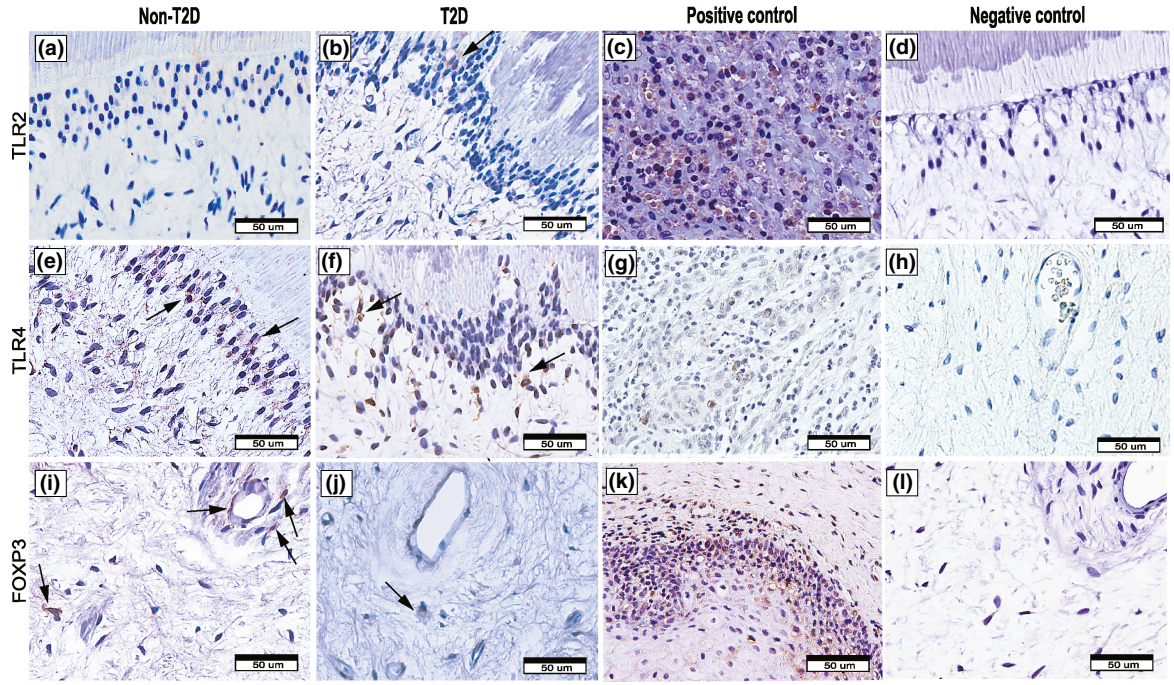

FIGURE 4 Photomicrograph showing immunolabelling pattern of anti-TLR2, anti-TLR4 and anti-FOXP3 from non-T2D and T2D participants, and positive and negative control samples. Anti-TLR2 staining was only very weakly detected in the odontoblast region (a and b) while moderate anti-TLR4 staining was found (e and f). Strong anti-FOXP3 staining was observed for non-T2D samples around blood vessels (i) while in the T2D samples staining was weak (j). Black arrows indicate positive staining. Lymph node tissue was used as the positive control for all antibodies and non-specific monoclonal IgG was used as a negative control (a–l; 40× magnification, scale bar 50 μm)

Stabley & Towler, 2017). These findings suggest a similar response in the human pulp.

Clinically, calcifications frequently cause challenges performing root canal treatment and increase the risk of procedural errors (Goga et al., 2008). Although there have been reports indicating that intraoral radiographs may assist in the preoperative diagnosis of calcifications, most studies have been retrospective case series and clinical experience suggests imaging is not always reliable preoperatively (Hsieh et al., 2018). The presence of calcifications, irregular dentine and degenerative neural tissue observed indicate that these morphological changes associated with T2D may diminish dental pulp sensitivity, resulting in asymptomatic pulpitis and in part explain the clinical challenges in accurately diagnosing the health of the pulp from patient history and sensibility tests. Furthermore, misdiagnosis is more likely which in turn influences clinical decision-making, outcome and prognosis. The use of cone-beam computer tomography has not previously been indicated for this group of patients but may be beneficial when findings are inconclusive.

Type 2 diabetes may adversely affect pulp healing following deep caries or VPT. This has been demonstrated in animals following pulp capping with MTA (Garber et al., 2009) and can be attributed to reduced vascularity. In addition, this study suggests that fewer fibroblast-like cells necessary for healing increased dense collagen, and pro-inflammatory factors are also likely to contribute to impaired or delayed healing. Indeed, this aligns to the poorer healing outcome following root canal treatment in adults with T2D (Nagendrababu et al., 2020). Whilst T2D is normally diagnosed in adults and age is not a contraindication to the use of VPT (Awawdeh et al., 2018), the findings of the current study highlight the need for chairside tests that can accurately diagnose the inflammatory status of the pulp. Furthermore, it suggests that T2D may influence the outcome of VPT, and prospective clinical studies are warranted.

Altered immune-inflammatory responses at local and/or systemic levels are well recognized in T2D (Berbudi et al., 2020). T2D was associated with increased expression of immune cells and pro-inflammatory cytokines within the pulp. Protein markers for dendritic cells (CD83) and tissue macrophages (CD68) were significantly upregulated in the T2D samples. Macrophages are immunosurveillance cells with key roles in host defence and homeostasis where they have important functions in eliminating irritants and pathogens to promote healing. In T2D, these cells are known to have a reduced capacity for defence and tissue repair. The reason for this is not well understood but has been attributed to metabolic reprogramming where high glucose desensitises macrophages to cytokine

stimulation and reduces their phagocytic function (Pavlou et al., 2018). In this study, there was a twofold increase in CD68+ cells and significant increase in inflammatory cytokines (IL1β, IL6, IL17 and TNF-α) in the T2D pulp. The increase in IL17 is noteworthy because this cytokine acts in a feedback loop to sustain inflammation by the activation of IL1β, IL6 and TNF-α (Abdel-Moneim et al., 2018). Pro-inflammatory effects are central to the development of diabetic complications, and the current findings may suggest that similar dysregulation and pathological processes occur within the dental pulp.

The clinically normal dental pulp has a role in immunosurveillance and can elicit an early defence response to pathogens and irritants via the innate immune system. Hyperglycaemia leads to chronic activation and an unregulated innate immune response, which is known to contribute to diabetic complications (Graves & Kayal, 2008). Toll-like receptors are involved in the earliest phase of the host innate immune response and induce the expression of various immune and inflammatory genes via NF-κB stimulation (Farges et al., 2009; Mutoh et al., 2007). Although the number of TLRs expressed was small, they were significantly increased in T2D samples. In particular, the presence of TLR4 in the vicinity of odontoblasts confirms the importance of these cells in immunosurveillance for patients with T2D. A similar increase in TLRs in response to hyperglycaemia in gingival and periodontal tissues has been noted (Mutoh et al., 2007; Promsudthi et al., 2014). TLR2 and TLR4 are upregulated in the presence of dental caries (Farges et al., 2015; Takeda et al., 2003); however, the present study excluded these cases and indicates that hyperglycaemia may activate TLRs in dental pulp in the absence of microbial irritants.

Regulatory T cells have a central role in controlling inflammation by inhibiting T-cell proliferation and cytokine production. The current study found the T2D pulp had increased cytokine expression but decreased numbers of FoxP3+ Tregs to provide an anti-inflammatory effect. Furthermore, IL17 acts in the early stages of inflammation and inhibits the effects of FoxP3. Taken together, fewer Tregs and increased IL17 reduce the immunosuppressive effect and enhance the potential for diabetic complications. What remains unknown is the level of inflammation and immunosuppression that the dental pulp can withstand before its defences becomes overwhelmed.

This study allows us to better appreciate the relationship between diabetes and the dental pulp and has provided new knowledge to further understand the influence of T2D on the morphology and immune markers within the tissue. It indicates that T2D may result in similar changes within the pulp to other body tissues. Furthermore, it highlights the complexity of the inflammatory process within the pulp and offers a fresh perspective for clinicians when making clinical decisions related to performing vital pulp therapy and the potential for pulp healing. These early findings inform and raise questions about how the pulp may respond to irritants according to the systemic status of the patient.

CONCLUSION

Hyperglycaemia may result in similar complications in the human dental pulp to other body sites. The morphological and immune differences observed in T2D pulp samples resembled changes associated with ageing. Fibrosis, reduced vascularity, calcifications and pro-inflammatory effects within the pulp from T2D may modulate the response to pulpal irritants, infection and healing, and so these findings offer a new perspective which may inform the interpretation of clinical findings, prognosis and healing.

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No conflict of interest.

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AUTHOR CONTRIBUTION

Shaikhah Alsamahi: Contributed to design and completed all experimental aspects of the study, draft manuscript. **Trudy M. Milne:** Study design, Guidance in laboratory techniques and analysis, manuscript review. **Haizal Hussaini:** Immunohistochemistry guidance and analysis. **Alison M. Rich:** Immunohistochemistry guidance and manuscript review. **Lara T. Friedlander:** Primary supervisor, manuscript preparation and corresponding author.

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