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ORIGINAL RESEARCH

Presence of methanogenic archaea in necrotic root canals of patients with or without type 2 diabetes mellitus

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Abstract

Theoretically, a necrotic root canal fulfils all requirements as a niche for methanogens to inhabit. However, their presence in it and its implication in apical periodontitis (AP) is controversial. Therefore, to contribute to ending the controversy, this study aimed to detect and compare methanogens' presence in two distinct niches with supposedly different microenvironments; both were necrotic root canals associated with AP but one from patients with type 2 diabetes mellitus (T2DM) while the other from non-diabetic patients. A clinical examination was performed on 65 T2DM patients and 73 non-diabetic controls. Samples from necrotic root canals were obtained, and methanogens were identified. The presence of methanogens was three times higher (27.6%) in the T2DM group than in non-diabetic patients (8.2%). In addition, methanogens' presence was associated with a higher prevalence of periapical symptoms.

KEYWORDS

apical periodontitis, methanogenic archaea, methanogens, necrotic root canal, type 2 diabetes mellitus

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INTRODUCTION

The oral cavity carries multiple anatomical niches where physicochemical features, such as pH, oxygen, temperature or redox potential, influence the microenvironment and, as a result, the establishment of a complex microbiota with a wide variety of microorganisms that includes bacteria, fungi, viruses, protozoa and archaea. Besides, this microbial composition and diversity can be altered by systemic conditions such as diabetes mellitus (DM) [1, 2]. Members of the domain *Archaea* are conspicuously underrepresented [3] and constitute only a minor part of the oral microbiota. They are restricted to a few species (*Methanobrevibacter*, *Methanosphaera, Thermoplasmatales*, *Methanobacterium* and *Methanosarcina*), all methanogens [4].

Although methanogens are now recognised as a component of the human microbiota, none has been established as an evident causative agent in human disease. However, they share some features with known pathogens that may reflect the potential to cause disease. Such features include ample access to a host, capabilities for long-term colonisation and coexistence with endogenous microbiota [5]. The only report that gives evidence of the possible pathogenic significance of a methanogen examined the serum immunoglobulin G responses to its components, indicating that antigenic molecules could act as modifiers or even initiators of inflammatory processes [6]. In this sense, methanogens are considered emerging periodontal pathogens because their detection has been frequent in patients with periodontitis [6], especially in deep periodontal pockets. Also, their abundance correlates to the severity of periodontal disease, and a reduction in their quantity has been notable after periodontal treatment [2, 5, 6].

Otherwise, methanogens have been associated with other oral infections, such as periimplantitis [7] and apical periodontitis (AP) [5, 8]. The infectious aetiology of AP and the central role of microbial factors in the initiation, development and persistence of this condition have been widely documented [9]. Diverse studies have shown that bacteria are the leading causative agent. However, some data suggest that other microorganisms, such as fungi, especially *Candida albicans* [10] and viruses [11], can cause pulp tissue inflammation, necrosis and AP. In principle, the necrotic root canal microenvironment fulfils all requirements for methanogens to inhabit. There are several reports, even in oral healthy subjects, where methanogens are part of their tongue biofilm, supragingival biofilm or saliva [12]; from these zones, methanogens could easily migrate to an exposed root canal. However, methanogens presence in it is still controversial since some investigations have not found them [13, 14] some have reported them with a minor (3.3% [15], 6% [16]) or moderate (25% [17], 28.5% [8]) frequency while others in a very high frequency (45% [5] and up to 85% [18]). Hence some conclude that they are not present in the root canal nor implicated in AP [14], while others consider it intimately connected [17]. The vast differences in these studies may be due to the root canal microenvironment mainly determined by the characteristics of each population because environmental factors have been reported as critical for methanogens variation [19]. Therefore, to provide information that contributes to ending the controversy, this study aimed to detect and compare methanogens' presence in two distinct niches with supposedly different microenvironments; both were necrotic root canals associated with AP but one from patients with type 2 diabetes mellitus (T2DM) while the other from non-diabetic patients.

MATERIALS AND METHODS

Patient population and clinical evaluation

This prospective cross-sectional observational study included 138 patients who requested endodontic services at the Department of Endodontics of the Faculty of Medicine at the Autonomous University of Querétaro. Participants were divided into two groups: 65 T2DM patients previously diagnosed according to the American Diabetes Association (by the presence of two or more of the following criteria or one but repeated on different days: glycated haemoglobin >6.5%, fasting blood glucose >126 mg/dL, random blood glucose >200 mg/dL) and 73 non-diabetic patients as the control group. The protocol study was approved by the Ethical Committee of the Dentistry Department of the Faculty. Informed and voluntary consent was obtained before the clinical examination, according to the ethical principles of the Declaration of Helsinki (version 2013).

A resident of the endodontic specialisation programme performed the clinical examination; in order to identify the pulp status, a thermal testing was done (Endo-Ice, Hygenic) while to determine the periapical status, vertical and horizontal percussion of the tooth and adjacent teeth were performed, as well as palpation of underlying tissues. Thus, the presence of swelling, tooth mobility, pain and tenderness through percussion or palpation were evaluated. Only teeth diagnosed with pulp necrosis and AP with a good quality coronal restoration potential were included.

Pregnant or lactating patients and those who suffer any other systemic disease, marginal periodontitis (determined when the pocket depth was >3 mm, and the attachment loss was ≥2 mm in at least 30% of the measured

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sites [20]), sinus tract and smokers or who have received antibiotics in the last 3 months were excluded. Prior to any procedure, a fast blood glucose test was performed on all patients to guarantee treatment safety (a maximum of 300 mg/dL in T2DM patients) and exclude possible diabetics from the control group (a maximum of 126 mg/dL).

Sample collection

Local anaesthesia was administrated with 2% lidocaine HCI with 1:100 000 adrenaline (Lignospan Standard, Septodont). The main and adjacent teeth were cleaned and disinfected with 3% hydrogen peroxide. A rubber dam was positioned, caries and coronal restorations were removed, and coronal access with sterile carbide burs was done. The operative field was cleaned with a cotton ball immersed in a 2.5% sodium hypochlorite (NaOCl) solution.

A number 3 Gates Glidden drill was used for coronal flaring with 2.5% NaOCl solution irrigation, which was then neutralised with 5% sodium thiosulfate solution (Sigma Chemical Co.). Root canal samples were obtained with a new, sterile, size 15 stainless-steel hand file, introduced into the root canal while determining tentative working length with a Root ZXII electronic apex locator (J. Morita). The hand file was then introduced into a microtube containing sterile phosphate-buffered saline (PBS) and vortexed. Two sterile size 15 paper tips were consecutively introduced into the root canal at the same length to absorb the fluid for 30 s and transferred to the same microtube. In the case of teeth with wide root canals (e.g. incisors), larger diameter files were used to determine the tentative working length, and this same file was used to take the sample along with paper tips of the same diameter. The root canal treatment was routinely continued, and the sample was kept at −80°C until deoxyribonucleic acid (DNA) extraction.

DNA extraction and polymerase chain reaction (PCR)

Microtubes with the samples were centrifuged (16 000 rpm for 10 min) to obtain the cell pellet. After removing the supernatant and paper tips, the pellet was washed three times with 1 mL of PBS (pH 7.4) and resuspended in 200 mL of cell lysis buffer (1.0% Triton X-100, 20 mM Tris–HCl, 2 mM EDTA, pH 8.0) and incubated at 85°C for 15 min. Then, 100 mL of 200 U/mL mutanolysin (Sigma) was added and incubated at 50°C for 1 h, followed by treatment with 80 mL of a second cell lysis solution (Puregene DNA isolation kit, Gentra Systems) at 80°C for 15 min. 100 mL of protein precipitation solution (Puregene DNA isolation kit, Gentra Systems) was added, and the proteins were removed by centrifugation (16 000 rpm for 10 min). The DNA was purified by phenol–chloroform–isoamyl alcohol (25:24:1, v/v; Invitrogen) extraction and isopropanol precipitation. The extracted DNA was dissolved in 50 mL of sterile molecular biology-grade water.

Specific oligonucleotide primers were used to identify the functional gene *mcrA* encoding for methyl-coenzyme M reductase, a key enzyme involved in methanogenesis that has been widely used for methanogens identification; a forward primer, 5′GGTGGTGTMGGATTCAC ACARTAYGCWACAGC-3′ and a reverse primer, 5′-TTCATTGCRTAGTTWGGRTAGTT-3. PCR assays were carried out using the following cycling parameter: initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 45 s, annealing at 55°C for 45 s and 72°C for 1 min 30 s, followed by a final extension at 72°C for 7 min. Positive and negative controls were included in each assay. Amplicons were analysed by electrophoresis in a 2% agarose gel and a 100-bp DNA ladder marker. Each gel was stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

All quantitative data were expressed as mean, standard deviation and range. Qualitative data were expressed as frequency and proportion. Statistical differences between groups in quantitative variables were determined by Student's *t*-test and ANOVA because of the normality of the data distribution. The chi-square test and Fisher's exact test for qualitative variables. GraphPad Prism V3.0 (GraphPad Software) was used, and statistical significance was established at $p < 0.05$. Statistical power was calculated with PS: power and sample size calculation software (HyLown Consulting).

RESULTS

The clinical characteristics of the patients are summarised in Table 1. Although the female was predominantly in both groups, there was no statistical difference in the distribution of the patients by gender or age ($p > 0.05$). Neither statistical difference was present in the distribution of the included teeth nor their clinical characteristics ($p > 0.05$), except for tenderness to percussion, which was present with higher frequency in the T2DM group ($p = 0.0339$). Furthermore, the presence of methanogens was significantly higher ($p = 0.0032$) in

TABLE 1 Clinical characteristics of the included patients and teeth in each group.

Group	Control ($n=73$)	T2DM ($n=65$)	p-Value
Mean ± SD (range)			
Age (years)	45.11 ± 13.17 (22–74)	48.46 ± 11.33 (28–69)	0.1135[a]
Frequency (%)			
Female	49 (67.1)	51 (78.4)	0.1814[b]
Male	24 (32.8)	14 (21.5)	
Incisors	15 (20.5)	16 (24.6)	–
Premolars	15 (20.5)	19 (29.2)	0.3070[c]
Molars	43 (58.9)	30 (46.1)	–
Caries	58 (79.4)	48 (73.8)	0.5450[b]
Restoration	51 (69.8)	41 (63.0)	0.4703[b]
Presence of pain	53 (72.6)	56 (86.1)	0.0610[b]
Tenderness to palpations	49 (67.1)	50 (76.9)	0.2564[b]
Tenderness to percussion	41 (56.1)	48 (73.8)	0.0339[b,d]

Abbreviations: SD, standard deviation; T2DM, type 2 diabetes mellitus.

[a]Student's t-test.

[b]Fisher's exact test.

[c]Chi-square test.

[d]Statistical significance.

TABLE 2 Frequency and proportion of methanogens in each group.

Group	Control ($n=73$)	T2DM ($n=65$)	p-Value	Power, 1-β[b]
Frequency (%)				
Presence of methanogens	6 (8.2)	18 (27.6)	0.0032[a]	0.9169

Note: Fisher's exact test.

Abbreviation: T2DM, type 2 diabetes mellitus.

[a]Statistical significance.

[b]Type I error rate, α 5%.

this group (Table 2); also, when performing the statistical power analysis, it was observed that a 90% power with a confidence level of 95% was obtained with the sample size. When grouping according to methanogens presence, the frequency of tenderness to percussion remained statistically higher ($p=0.0221$) in the T2DM group (Table 3).

DISCUSSION

DM is a metabolic disease characterised by hyperglycaemia that results from defects in insulin secretion, insulin action or both. This systemic disease induces changes in immune cell function and produces an inflammatory immune cell phenotype predisposing to chronic inflammation, progressive tissue breakdown and diminished tissue repair capability. Also, since the immune system plays a crucial role in response to infection, patients with poor glycaemic control are prone to severe and recurrent bacterial or fungal opportunistic infections [21, 22]. It has been reported that DM patients also exhibit an increased prevalence of oral diseases, such as mouth dryness, periodontal disease, swelling of the parotid glands, oral candidiasis and AP [23]. For this last, it is known that DM influences its development, course and response to the treatment. Even poorer treatment outcome has been observed for DM patients, and the rate of flare-ups has been about twice as high as in non-diabetic patients [24, 25]. Also, modifications in the oral microbiome diversity have been reported [1, 2]. Because of these well-known precedents, the necrotic root canal of T2DM patients could represent a distinct niche in which a different microenvironment is present due to the systemic condition. The major presence of methanogens in these niches and its less presence in the non-diabetic niches provide evidence of its presence in necrotic root canals with specific physicochemical features.

The main strength of this investigation relies precisely on comparing distinct niches of two groups of patients from the same population, thus avoiding geographic, ethnic, nutritional, socioeconomic and other differences that are present if we want to compare the results between all other previous studies. The population of different countries may likely exhibit different methanogen prevalence since environmental factors play a role in the repertoire variation of methanogens [19]. A clear example of this

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TABLE 3 Clinical characteristics of control and T2DM groups divided by the presence of methanogens.

Group	Control without methanogens ($n=67$)	Control with methanogens ($n=6$)	T2DM without methanogens ($n=47$)	T2DM with methanogens ($n=18$)	p-Value
Mean ± SD (range)					
Age (years)	45.0 ± 13.1 (22–74)	46.0 ± 14.5 (26–62)	44.3 ± 10.0 (28–62)	59.1 ± 6.6 (46–69)	<0.0001[a,c]
Frequency (%)					
Female	45 (67.1)	4 (66.6)	37 (78.7)	14 (77.7)	0.7290[b]
Male	22 (32.8)	2 (33.3)	10 (21.2)	4 (22.2)	
Incisors	14 (20.8)	1 (16.6)	13 (27.6)	3 (16.6)	0.6665[b]
Premolars	13 (19.4)	2 (33.3)	14 (29.7)	5 (27.7)	
Molars	40 (59.7)	3 (50)	20 (42.5)	10 (55.5)	
Caries	53 (79.1)	5 (83.3)	31 (65.9)	17 (94.4)	0.0924[b]
Restoration	46 (68.6)	5 (83.3)	28 (59.5)	13 (72.2)	0.6869[b]
Presence of Pain	48 (71.6)	5 (83.3)	39 (82.9)	17 (94.4)	0.4577[b]
Tenderness to palpitations	49 (73.1)	5 (83.3)	34 (72.5)	16 (88.8)	0.9380[b]
Tenderness to percussion	37 (55.2)	4 (66.6)	31 (65.9)	17 (94.4)	0.0221[b,c]

Abbreviations: SD, standard deviation; T2DM, type 2 diabetes mellitus.

[a]ANOVA.

[b]Chi-square.

[c]Statistical significance.

can be noticed in the two studies in which the highest frequency of methanogens in root canals has ever been reported, both were done in Polish population [5, 18], while two studies reported 0% and 3.3% in Brazilian population [14, 15]. In addition, another strength of this investigation is that there were no differences in the distribution by age or gender, and both groups had strict selection criteria. Tooth diagnosis was the same; there were no differences in the type of tooth nor in its clinical history by caries or restorations. Thus, the presence of T2DM was the primary variable between groups.

Additionally, although it was not the objective of this study, the results also provided information regarding the role that methanogens could be playing when present in the necrotic root canals since it was clear that they were associated with a more virulent microbiota, as has already been suggested in periodontitis and periimplantitis in where its presence was associated with greater severity of the diseases [7, 8]. In this study, they were associated with a higher frequency of tenderness to percussion, in addition to higher reported pain and tenderness to palpitation, although the two last were not statistically significant. This coincides with a previous report that associated symptomatic patients with methanogens in the root canal [8]. It cannot be confirmed that methanogens are directly involved in this augmented periapical inflammation. Still, it gives support to the hypothesis that has been raised by others, which states that methanogens have a role as a keystone driver of metabolic support [5, 26] to pathogenic bacteria by increasing their activity through alteration of the microenvironment in the root canal and contributing to local apical tissue damage.

Various syntrophic and antagonist partners to methanogens have been proposed. Horz et al. [27] found a significant positive correlation between methanogen abundance and the amount of *Prevotella intermedia*. The *Prevotella* genus is widespread in necrotic root canals, and its frequency is increased in DM patients since it is involved in the metabolic utilisation of carbohydrates; the abundance of *Prevotella* species occurs in response to an increase in carbohydrate intake [28]. In addition, they are hydrogen producers, and the fact that methanogenesis is a hydrogen-consuming process, allows their growth; this could be one of the specific physicochemical features of the root necrotic canal niche in T2DM patients.

On the other hand, the *Treponema* genus has been proposed as an antagonist because it is a hydrogen competitor that would be able to exclude methanogens from the environment [29]. This might partly explain the presence of methanogens in some but not all cases. Some studies have demonstrated that several *Treponema* species are present in different root canal infections. Some are highly prevalent, particularly *T. denticola*, *T. socranskii* and *T. maltophilum* [30]. Hence, their abundance and frequency in root canals have to do with a low prevalence of methanogens [18, 29]. Unfortunately, there is no information on the

Treponema genus in DM patients; possibly, it is present less frequently in them. Therefore, there is no competition with methanogens, and as a result, there is more probability of finding methanogens in these niches.

One of this study's significant limitations is that the microenvironment and microbiota of each niche (root canal) were not directly studied, so it cannot know what precise conditions or microorganisms were present in each one. Also, detailed data on glucose control for each patient is missing, which is also an important limitation in this study. These unknown variables could explain why some T2DM patients did not harbour methanogens. Since all T2DM patients were within the maximum glucose concentration to receive attention in the university clinic (300 mg/dL), it could be speculated that most T2DM participants were well glucose controlled; hence, their niches were not widely different from the non-diabetic patients. On the other side, the few non-diabetic patients presenting methanogens could have had a hidden local or systemic condition that compromised their immune system, which would result in an altered niche too. Even with this limitation, the significant increase of methanogens (more than threefold) in the necrotic root canals from T2DM patients provides evidence to support the hypothesis that methanogens' presence depends on the microenvironmental conditions of the niche. They are not essential for the development of pulp necrosis or AP; therefore, they are only present when physicochemical features of the niche allow it.

Clinical and experimental studies are missing to investigate the exact role of methanogens in the polymicrobial community of the root canal niche. However, at present, methanogens' presence could be used as a marker of a particular type of microenvironment related to a more virulent microbiota that can cause increased inflammation and pain.

In conclusion, methanogens could be present in necrotic root canals associated with AP, but they are not a common component nor essential to its development. However, its presence is significantly augmented in T2DM patients and was associated with a higher prevalence of periapical symptoms.

AUTHOR CONTRIBUTIONS

All authors have contributed significantly and are in agreement with the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors deny any conflicts of interest related to this study. They have no financial or nonfinancial interests to disclose.

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