Comparative expression of periodontal pathogens in pregnant women with periodontitis and diabetes in Indian population – A case-control study

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Abstract:

Introduction: This study compares two bacterial expression (Prevotella intermedia, Prevotella nigresens) and their expression in pregnant and gestational diabetic patients with periodontal disease and proposes to explore the microbiologic nature of the association between early colonizers, such as P.intermedia and P.nigresens are bound by later colonizers such as P. gingivalis and T.forsythia.

Materials and methods: Forty pregnant women in each group with Chronic Periodontitis with and without gestational diabetes, were grouped into two groups – as a case control study. Plaque samples from these patients were collected using paper points. The samples were evaluated using Real Time –Polymerase Chain Reaction technique.

Results: This study resulted in an increased presence of early colonizers in both groups, showing a "3 fold increase" of Prevotella intermedia among pregnant patients with diabetes and periodontal disease and showed a "1 fold increase" among pregnant patient patients without diabetes compared to Prevotella nigrescens.

Conclusion: The current study indicates that Prevotella intermedia is significantly increased among the gestational diabetic and therefore involved in the demonstrated microbial shift during pregnancy and Gestational Diabetes Melitus and more prone for periodontal breakdown.

Keywords: Chronic Periodontitis, Gestational diabetes, Prevotella intermedia, Prevotella nigrescens Tanerella forsythia

I. Introduction

The development of periodontal disease is a highly communicative and interactive process between pathogenic components in the dental plaque, the host tissues (including epithelium), the vasculature, immune systems, the connective tissue cells and their matrix.

More than 500 species of microorganisms have been isolated from periodontal pockets. Socransky's^[1] data suggest that the colonization of "orange" complex bacteria precedes colonization of "red" complex bacteria (Fig 1). There are conflicting scientific data on which of the Orange complex bacteria is present in a greater titre which necessitates the evaluation of the expression of the two phenotypically identical species, P.intermedia and P.nigrescens in pregnancy and diabetes.

Diabetes has been associated with increased prevalence and severity of gingivitis. Poorly controlled diabetes mellitus subjects had significantly greater bone loss and attachment loss, than did well controlled diabetes mellitus subjects. Periodontal diseases and diabetes mellitus are closely associated and the understanding of the relationship between periodontitis and diabetes including the factors associated with coexisting synergies has been established.

Gestational diabetes mellitus is defined as either onset or first recognition of glucose intolerance during pregnancy^[2]. Bacterial plaque has been established as the primary etiological factor for the initiation of periodontal disease. Hormonal changes during Pregnancy and changing microflora in Diabetes have been suggested as important

modifying factors that may influence the pathogenesis of periodontal diseases. Clinical periodontal disease has been previously associated with gestational diabetes mellitus in cross-sectional *and* case-control studies^[3,4] This study addresses which of the two orange complex bacteria is expressed in a greater titre in pregnant and gestational diabetic patients with periodontal disease



Figure 1 : Microbial complexes in subgingival plaque

II. Materials And Methods

II.1 Subject Selection

The 80 patients who had taken part in the present study were chosen from among the patients who attended the Dept of Diabetology at Government General Hospital, Madras Medical College, Chennai.

Group A - consisted of forty Pregnant women with Gestational Diabetes with Chronic Periodontitis. **Group B** - consisted of forty Pregnant women without Gestational Diabetes with Chronic Periodontitis

II.2 Selection Criteria

Inclusion Criteria –

(Criteria A and B were common to both groups)

- Age: 20-30 years.
- Diagnosed as chronic periodontitis and having atleast 2 sites with ≥5mm of periodontal pocket and associated ≥ 3mm attachment loss and with no associated gingival recession.
- Gestational diabetes group (Group A)
- -Patients diagnosed with gestational diabetes and in the 2nd trimester of pregnancy
- Non-Gestational diabetes group (Group B)
- -Patient systemically healthy in the 2nd trimester of pregnancy

Exclusion Criteria -

- Patients who had received periodontal therapy in last six months.
- Patients who had received antibiotics in the last six months.
- Patients with any other systemic diseases like hypertension, thyroid disorders and any diagnosed hormonal disorder.
- Patients who were smokers or alcoholics

The study was approved by the Institutional Ethical committee, Patients included in the study were explained about the study and written informed consent was obtained.

II.3 Criteria for Assessment of Gestational Diabetes:

Gestational diabetes was assessed based on the lab report obtained from the hospital. Patients were given 100mg of oral glucose and their blood sugar levels were evaluated at baseline (fasting), 1 hour, 2 hour and 3 hours. If 2 of the 4 values in the Glucose Tolerance Test were at or above the cut-off levels (fasting glucose >95 mg/dl, one-hour glucose > 180 mg/dl, two hour glucose > 155 mg/dl, or three-hour glucose > 140 mg/dl), then it was diagnosed as gestational diabetes mellitus^[5]

11.4 Periodontal Status Evaluation:

Clinical parameters assessed for the study were oral hygiene index – simplified, plaque index, modified sulcular bleeding index, probing pocket depth and clinical attachment level.

II.5 Criteria For Site Selection:

Sites for plaque collection in both the groups were based on the following criteria-Presence of bleeding on probing, clinical attachment loss $\geq 3mm$ and probing depth $\geq 5mm.(Fig 2)$

II.6 Procedure For Sample Collection:

Subgingival plaque samples were collected from the mesial and buccal sites of teeth with the deepest pocket by means of a sterile paper points (# 35, US Patent no - 5,833,458)(Fig 3, Fig 4). Samples were placed in 0.1 mL Ethanol (99.9% pure, M.W. 46.08). After all the samples were collected, the samples were analysed for quantification of periodontal pathogens by using the REAL TIME PCR. The processing reagent, PCR reagents and Master Mix Kit were obtained from Applied Biosystems, Warrington, USA.

II.7 Processing Of Samples:

Collected plaque samples were stored in Eppendorf tubes containing Ethanol solution (99%) at -80°C and processing was carried out by m-RNA isolation. After a series of processing the resulting supernatant was discarded and the remaining pellet was dried for 2 hrs and add 30 μ l sterile water was added to it, then freezed and sent for PCR analysis.(Fig 5)



Figure 2 - Criteria For Site Selection



Figure 4-Sample Collection



Figure 3 - Paper Points



Figure 5 – RT – PCR Analysis

II.8 Quantification Of Periodontal Pathogens Using PCR Analysis.

To setup PCR reactions, commercially available Sybrgreen master matrix(Applied Biosystems) - Reverse primer -0.5μ l, Template DNA -2μ l, sterile water -2μ l was added. The total 10ml of the mix is dropped in micro wells. Then the micro wells were kept for PCR analysis in the PCR machine.

Amplification is done by the values obtained from delta Ct. Lower the value, higher the expression of periodontal pathogens. In the current study, the amplification of RT-PCR is done using relative quantification which relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples.

II.9 Statistical Analysis :

Mean and standard deviation was estimated from the sample for each study group. Mean values were compared among different study groups by using independent t test with p<0.001(99.9% sig) was considered as the level of Statistical Significance. The statistical software SPSS was used for the analysis of the data.

III. Results

A total number of eighty subjects comprising of forty pregnant women with Gestational diabetes and co-existent Chronic Periodontitis and forty pregnant women with Chronic Periodontitis and without Gestational Diabetes. This study apart from showing an increased presence of Orange Complex , showed a "3 fold increase" of P. intermedia among pregnant patients with diabetes and periodontal disease and showed a "1 fold increase" among pregnant patient patients without diabetes compared to P.nigrescens the by $(\Delta\Delta Ct = \Delta Ct - \Delta Ct)$ method. (Tables 1 to 5) (Graph 1, Graph 2 and Graph 3)

					Std. Error
	SUB GROUP	N	Mean	Std. Deviation	Mean
Ct	Control	40	23.7960	1.54513	.24431
	GDM	40	21.5957	1.48740	.23518

Table 1 : Independent T-Test Comparing Bacterial Load : Group Statistics p < 0.001(99.9% sig),

		Levene's Equality of	Test for Variances	t-test for Equality of Means							
							Mean	Std. Error	95% Confidence Interval of the Difference		
		F	Sig.	t	df	Sig. (2-tailed)	Dif f erence	Dif f erence	Lower	Upper	
Ct	Equal variances assumed	.102	.750	6.488	78	.000	2.20025	.33911	1.52514	2.87536	
	Equal variances not assumed			6.488	77.887	.000	2.20025	.33911	1.52512	2.87538	

Table 2 : Independent sample Test comparing bacterial load p<0.001(99.9% sig)

		Levene's Equality of	Test for Variances	t-test for Equality of Means								
							Mean	Std. Error	95% Cor Interva Diffe	nfidence I of the rence		
		F	Sig.	t	df	Sig. (2-tailed)	Dif f erence	Dif f erence	Lower	Upper		
Ct	Equal variances assumed	.015	.902	9.593	78	.000	2.63850	.27505	2.09092	3.18608		
	Equal variances not assumed			9.593	77.956	.000	2.63850	.27505	2.09092	3.18608		

Table 3 : Independent sample "For P. intermedia" p<0.001(99.9% sig)

		Levene's Equality of	Test for Variances	t-test for Equality of Means							
							Mean	Std. Error	95% Confidence Interv al of the Difference		
		F	Sig.	t	df	Sig. (2-tailed)	Difference	Dif f erence	Lower	Upper	
Ct	Equal variances assumed	9.528	.003	6.445	78	.000	1.27700	.19813	.88255	1.67145	
	Equal variances not assumed			6.445	67.273	.000	1.27700	.19813	.88155	1.67245	

Table 4 : Independent sample Test "For P. intermedia"p<0.001(99.9% sig)</th>

		Levene's Equality of	Test for Variances	t-test for Equality of Means								
							Mean	Std Error	95% Confidence Interv al of the Difference			
		F	Sig.	t	df	Sig. (2-tailed)	Difference	Difference	Lower	Upper		
Ct	Equal variances assumed	11.096	.001	-5.558	78	.000	-1.24925	.22475	-1.69669	80181		
	Equal variances not assumed			-5.558	60.735	.000	-1.24925	.22475	-1.69870	79980		

Table 5 : Independent sample -Test Comparison of P. intermedia Vs P. nigrescens p < 0.001(99.9% sig)



Graph 1 : fold increase of bacteria - In GDM patients there is a "3 fold increase" in P.intermedia Vs P.nigrescens In non GDM patients there is a "1 fold increase" in P.intermedia Vs P.Nigrescens



Graph 2: The Real Time PCR Amplification Plot (for P.intermedia)Gives a amplification plot of P.intermedia showing GDM group plot.



Graph 3: The Real Time PCR Amplification Plot (for P. nigrescens) Gives a amplification plot of P. nigrescens showing GDM group plot.

IV. Discussion

The state of periodontal disease can be defined as an imbalance between the quality and quantity of bacterial microflora colonizing the periodontal pocket and the immunological potential of the host, which can be modified by several risk factors.

Despite the widespread acceptance of the specific plaque hypothesis^[6] in the etiology of chronic periodontitis, periodontal pathogens are frequently detected in periodontally healthy individuals^[7]. Nevertheless, once formed, deep periodontal pockets can provide a suitable environment that further selects specific anaerobic bacterial complexes^[8]. Factors that alter this subgingival environment, include inflammation and the myriad of immune and metabolic factors that can influence the composition of the subgingival biofilm.

In this context, factors such as diabetes that alter the nature of the immune/inflammatory response could conceivably influence which bacterial complexes form subgingivally. While certain bacterial species are more commonly found in diabetic patients, it is more difficult to determine whether this occurs because of direct alterations to the subgingival microenvironment or whether it occurs indirectly by alterations to the host response. Diabetic individuals may be more susceptible to chronic periodontitis as a result of hyperglycemia altering the subgingival microenvironment such that bacterial species that are more pathogenic in nature will become dominant.

Clinical periodontal disease has been previously associated with Gestational Diabetes Mellitus in cross-sectional studies. Novak *et al*^[9] in a cross-sectional study of 4244 pregnant women found that those with gestational diabetes (113 women) had a much higher prevalence of periodontal disease. Their study further found that this increase in periodontal disease was associated with an increase in the levels of dental plaque.

Xiong *et al* ^[3], in another cross-sectional study of 53 pregnant women with gestational diabetes and 106 pregnant women without Gestational Diabetes found that 77.4% women with Gestational Diabetes had periodontitis, whereas only 57.5% of the women without gestational diabetes had periodontitis. Their results indicated a statistically significant association of periodontitis to Gestational Diabetes mellitus.

A.P. Dasanayake ^[10], tried to correlate the microflora from subgingival microflora procured from pooled samples from first molar teeth, to the overall periodontal status of the patient. He then further tried to correlate subgingival plaque samples with microbiological samples from the vagina and cervix. The study was proposed to assess if altered periodontal status in a gestational diabetic patient could alter pregnancy outcomes. The Dassanayake study ^[10] was however unable to make a correlation between oral subgingival microflora and cervical or vaginal microflora.

There has been conflicting data on the importance of orange complex bacteria at the site, in pregnant patients with periodontitis who either have or do not have gestational diabetes. One study done by Gursoy et al 2012 ^[11] proposed that pregnant women harbor increasing numbers of P.nigrescens at sites with periodontitis while another study proposed that there is significantly higher frequency of P.intermedia among the diabetic pregnant women when compared with P. nigrescens, indicate an increased risk for future periodontal disease . This is in contrast to earlier studies which in indicated that P. intermedia is increased among pregnant patients with gingival and periodontal disease

In the present study, P.Intermedia, expression was higher in the Gestational group with Chronic Periodontitis (24.9 ± 0.19) ng/ml when compared to the Non-Gestational group with Chronic Periodontitis (27.3 ± 0.19) ng/ml. This also showed a statistically highly significant difference (p<0.0001) between Gestational Diabetic Group and Non-Gestational Diabetic Group with Chronic Periodontitis.

The intra group comparisons in the Gestational Diabetes Group revealed Cycle threshold levels of P.nigresencs was at a much lower level on comparison with P.intermedia with their difference being statistically highly significant (p<0.0001). A similar difference was noted in the non Gestational Diabetic group.

Though both the groups had higher levels of P.intermedia, the expression in the Gestational Diabetic group was significantly higher. It remains to be evaluated whether this is due to an increase in glycemic levels. This study also found increased amount of plaque score in the gestational diabetic group when compared to the non Gestational Diabetic group. This difference though in contrast to the study by Gutthmiller^[12], was not statistically significant (p<0.025).

The conflicting scientific data on which of these Orange complex bacteria is present in a greater titre in the sub gingival plaque of pregnant patients with periodontal disease necessitates this study to address which of these bacteria is expressed in a greater titre in pregnant and gestational diabetic patients with periodontal disease.

Coaggregation has been suggested to be a critical colonization strategy of oral microorganisms, involving numerous surface ligands and receptors, which have been suggested to help instruct the construction of the complex biofilms. Investigators have noted Earlier colonizers, such as P.intermedia are bound by later colonizers such as P. gingivalis and T. forsythia^[13] Later colonizers are often strict anaerobes that increase in plaque when a more anaerobic environment develops, which may be due, in part, to the actions of earlier colonizers. Partner cell type specificity is also observed in pairwise coaggregations with species associated with periodontal disease.

This study could have been carried on until post partum, to assess for possible adverse pregnancy outcomes. This was not possible in this study as it might have resulted multiple patient drop outs from the study.

This study could have also correlated microbial factors to host derived inflammatory mediators which could have given a more comprehensive understanding on the pathogenisis of periodontal disease in Gestational Diabetics. This has not been attempted in this study as it is beyond the scope of a singular dissertation. This study on the other hand has given focus to an area of periodontal microbiology were there has been credible data, to relate to the observed increased periodontal destruction in Gestational Diabetics.

Socransky et al 1998^{[14],} showed that "orange" complex and "red" complex microorganisms were closely related. Bacteria in the "red" complex are rarely found in the absence of bacteria from the "orange" complex. Therefore the results of the present study agrees with of Socransky's hypothesis and treatment strategies can be aimed at treating the orange complex in patients with gestational diabetes and periodontitis which could be a crucial "missing link" in helping preventing or postponing the onset of red complex from occurring at a greater titre and thereby preventing greater magnitude of periodontal destruction.

V. Conclusion

Whether it is because of the effect of diabetes on subgingival plaque or the effect of the host response, that results in greater disease progression remains uncertain. Indeed, both mechanisms are probably involved. Likewise, the mechanism behind the effect of periodontitis on diabetic control is equally unclear. The importance of systemic inflammation exacerbated by periodontal inflammation is compelling. The role of bacteria in these processes has not been fully explored and remains the subject of much more future research.

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