ORIGINAL RESEARCH PAPER

Volume-7 | Issue-5 | May-2018 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

EVALUATION OF INCIDENCE OF PERIODONTAL PATHOGENS IN ATHEROMATOUS PLAQUES AND SUBGINGIVAL PLAQUES IN PATIENTS WITH PERIPHERAL VASCULAR DISEASES - A CLINICAL AND MICROBIOLOGICAL STUDY.

Periodontology

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ABSTRACT

Introduction: Peripheral artery diseases (PAD) and chronic periodontitis share common pathophysiology of chronic inflammatory diseases. Periodontal pathogens may play a role in the development and progression of atherosclerosis leading to vascular disease.So the present study was aimed to identify periodontal pathogens in atheromatous plaque and in sub-gingival plaque in patients with PAD .

Methodology : 30 patients with PAD were divided into Group A(15): PAD with healthy periodontium and Group B(15): PAD with chronic periodontitis. Subgingival plaque and atheromatous plaque samples were collected and subjected to Polymerase chain reaction analysis for the presence of pathogens.

Results : Group B samples reported higher levels of P.gingivalis (60%) in sub gingival samples and (46.7%) in atheromatous plaque .

Conclusion : Higher counts of P.gingivalis were reported in patients with chronic peridontitis and PAD thereby supporting the association between chronic periodontitis and PAD.

KEYWORDS

Vascular disease , periodontitis ,

INTRODUCTION:

Periodontal disease is a destructive disease of the supporting structures of the teeth, due to inflammatory processes induced by the microbial biofilm[1]. These pathogens enter the blood stream during transient bacteremia phase, where they undergo degradation of bacterial cell wall releasing lipopolysaccharide and endotoxin which leads to up-regulate the vascular cell function, endothelial cell dysfunction by inducing thrombus formation, vascular cell proliferation, apoptosis and cell death1 and produce measurable impact on the cardiovascular tissues.[2]

Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis causing a chronic, slowly developing, narrowing of the arterial vessels typically affecting the lower limbs and it has 20% of age related factor. Peripheral arterial diseases, chronic periodontitis and atherosclerosis[3] share common inflammatory pathophysiology and co-morbidity factors like smoking, diabetes, and hyperlipidemia increase the disease severity. Hence evidences have linked association between periodontal diseases and heart diseases.

Considering this potential oral infection-inflammatory pathway, the aim of the present study was to identify and correlate an incidence of periodontal pathogens in atheromatous plaques of peripheral arteries and subgingival plaques in periodontally healthy patients and patients with chronic periodontitis suffering from PAD.

AIMS AND OBJECTIVES

1. To evaluate the presence of periodontal pathogens in atheromatous plaque of peripheral arteries in patients with chronic periodontitis and periodontally healthy subjects.
2. To compare and correlate the presence of periodontal pathogens in atheromatous plaque and subgingival plaque in patients from both the groups.

MATERIALS AND METHODS

Source of data:This was a cross sectional study in which 30 patients aged 40-80 yrs and diagnosed with peripheral vascular diseases by CT Angiography and who required endarterectomy surgery were selected

Ÿ The study sample population were selected from the department of vascular surgery at Sri Jayadeva Institute of Cardiovascular science and Research Center Bangalore. Ethical clearance was obtained from the Institutional Review Board .An informed consent was obtained from the patients prior to the study.

INCLUSION CRITERIA

1. Patients diagnosed with PAD by CT Angiography and requiring endarterectomy surgery.
2. Periodontally healthy and chronic periodontitis patients.
3. Males and females patients aged 40-80 years.

EXCLUSION CRITERIA

1. Patients having major illnesses like malignancy, autoimmune diseases.
2. Patients who had undergone periodontal therapy during last six months.
3. Patient with a history of drugs intake like antibiotics, Immunosuppressive drugs or chemotherapy, three months prior to the study.
4. Patients with any other chronic systemic infections like tuberculosis.

Prior to the surgery periodontal examination was carried out for each patient and based upon the periodontal clinical parameters patients were divided into 2 groups.

Group A (n=15) - Patients with PAD and with healthy periodontium.

Group B (n=15)- Patients with PAD and with chronic periodontitis.

The following clinical parameters were recorded one day prior to the endarterectomy surgery.

1. Gingival Index(Loe and Sillness 1963).
2. Plaque Index (Sillness and Loe 1964) .
3. Sulcus Bleeding Index (SBI) (Muhlemann and Son 1971).
4. Probing Pocket Depth (PPD).
5. Clinical Attachment Level (CAL) .

Scoring is done based on the criteria. Probing pocket depth was measured from the free gingival margin to the base of the pocket using Williams graduated periodontal probe. The clinical examinations consisted of full-mouth recordings of six sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) for each tooth present. CAL was measured, as the distance from the cemento-enamel junction (CEJ) to the bottom of the clinical pocket.

COLLECTION OF PLAQUE SAMPLE

After assessing the clinical parameters, supragingival plaque was removed to prevent contamination of the flora, and then the area was isolated using sterile cotton rolls, dried and the subgingival plaque was collected using a sterile Gracey curette. The collected plaque was

transferred into labeled sterile eppendorf tube containing volume of 200µl of TE buffer (10mM Tris PH of 8.0, 1mM EDTA) for transfer and stored. Surgical specimen of peripheral artery atheromatous plaque was obtained during the endarterectomy procedure , and the entire specimen was placed in a vial containing volume of 20ml of TE buffer (10mM Tris PH of 8.0,1mM EDTA) for transfer and stored at -20°C till analysis. The subgingival plaque and atheromatous plaque samples were sent to the laboratory for conventional PCR analysis. PCR program was designed to suit each primers. The primers used for this study were specific for Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia. For samples with Porphyromonas gingivalis were preheated at 950C for 5 minutes, followed by amplification under the following conditions. Denaturationat 940Cfor 45 seconds,Annealing at 580C for 45 seconds and Elongation at 720C for 45 seconds.

Thirty cycles were followed by an elongation step at 720C for 10 minutes. Samples with Aggregatibacter actinomycetemcomitans were heated at 950C for 10 minutes, followed by amplification under the following conditions. Denaturation at 940C for 45 seconds, Annealing at 600C for 45 seconds and Elongation at 720C for 1 minute.

Thirty five cycles were performed followed by an elongation step at 720C for 10 minutes. (Four primers used one with 360 bp and 443bp, 641 base pairs, and 598 base pairs)Reactions were performed in duplicates for each sample, to exclude possibility of false negative results.

ANALYSIS OF PCR PRODUCT

20µl of amplified PCR product was loaded on 1 / 1.5 % Agarosegel.The gel was stained with propidium iodide and observed under ultra violet light transilluminator. 1 kb or 100 bp ladder (Fermentas) was used as molecular weight marker. The band position of PCR products was as mentioned in the literature.

BLOOD SAMPLE COLLECTION FOR ESTIMATION OF C-REACTIVE PROTEIN

2ml of venous blood was collected from the antecubital fossa of all the patients and transferred into anti-coagulant coated vacutainers. It was further sent to the laboratory for CRP analysis by turbidimetric immunoassay.

Statistical analysis

A subject level analysis was performed for each study parameter. Data was expressed by mean and standard deviations (SDs) for all variables by Student unpaired t test. The level of significance was set as p value < 0.05. The frequency of pathogen detected in atheromatous plaque in each patient with periodontal data was stratified according to gingival index (GI), Plaque index (PI), probing pocket depth (PPD), Clinical attachment level (CAL), sulcus bleeding index (SBI).Differences in terms of prevalence of pathogens in the atheromatous and subgingival plaque were determined by chi-square test. The increase prevalence percentage among the group A and group B was compared using Pearson's correlation test.

RESULTS

Table 1: Patient based data

Demographic Characteristics among study participants					
Sl.	Variables	Categories	Group 1	Group 2	P-Value
1	Age‡	[Mean ± SD]	43.1 ± 7.4	53.6 ± 10.7	0.005*
2	Sex n (%)‖	Males	14 (93.3%)	13 (86.7%)	1.00
		Females	1 (6.7%)	2 (13.3%)	

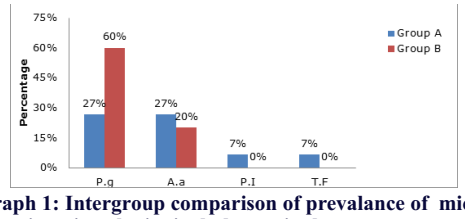
Table 2:COMPARISON OF CLINICAL PARAMETERS IN GROUP A AND GROUP B

Variables	Group	N	Mean	SD	S.E.M	Mean Differ	95% Cl of the Diff.		t	df	P-Value	
							Lower	Upper				
GI	Group A	15	0.40	0.51	0.13	-1.00	-1.43	-0.57	-4.778	28	<0.001*	
	Group B	15	1.40	0.63	0.16							
PI	Group A	15	1.00	0.00	0.00	-0.33	-0.59	-0.08	-2.646	28	0.01*	
	Group B	15	1.33	0.49	0.13							
PPD	Group A	15	3.00	0.71	0.18	-1.69	-2.18	-1.20	-7.035	28	<0.001*	
	Group B	15	0.33	0.49	0.13							
CAL	Group A	15	0.73	0.59	0.15	-2.00	-2.40	-1.60	-10.333	28	<0.001*	
	Group B	15	2.73	0.46	0.12							
SBI	Group A	15	1.07	0.23	0.06	-0.98	-1.22	-0.73	-8.150	28	<0.001*	
	Group B	15	2.05	0.40	0.10							

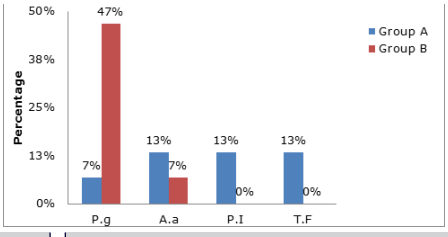
Table 3: COMPARISON OF CRP BETWEEN GROPUS

Groups	CRP	p-value
Group A	86.70%	0.48
Group B	100%	

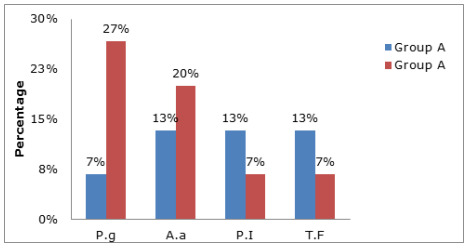
Graph 1: Intergroup comparison of prevalance of micro organisms in sub gingival plaque in between two groups



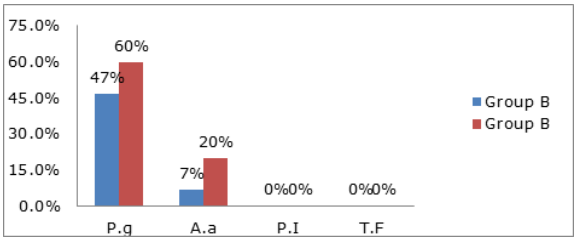
Graph 1: Intergroup comparison of prevalance of micro organisms in sub gingival plaque in between two groups



Graph 3: Intragroup comparison of prevalance of micro organisms in atheromatous and sub gingival plaque in Group A



Graph 4:Intragroup comparison of prevalence of micro organisms in atheromatous and sub gingival plaque in Group B



Total of thirty patients who underwent endarterectomy were divided into two groups. Mean age of the patients in Group A: 43.1 ±7.4yrs and in Group B is 53.6±10.3 was statistically significant with p-value of < 0.05 (table 1). The mean difference of all the clinical parameters when compared between group A and group B was statistically significant with p-value of <0.001(table 2). Estimation of C-reactive protein in Group A and Group B difference in the two groups was statistically not significant with p-value of 0.48 (table 3).

Periodontal pathogens were seen in both atheromatous plaque and subgingival plaque of all patients in both the groups. Patients having peripheral arterial diseases along with chronic periodontitis [Group B] reported higher levels of only P.gingivalis [60% in subgingival plaque, 46.7% in atheromatous plaque] other pathogenic bacteria were seen more prevalent in Group A patients (graph1 & 2).

The results of intragroup comparison of prevalence of pathogens in atheromatous plaque and subgingival plaque in group A using Pearson's correlation test were not statistically significant. The results of intragroup comparison of prevalence in group B using Pearson's correlation test were statistically significant with p<0.05 value in relation to P.gingivalis and A. actinomycetemcomitans (graph 3 & 4).

A.actinomycetemcomitans was found to be 20% in subgingival plaque, 6.7% in atheromatous plaque. T. forsythia and P.intermedia [13.3% in subgingival plaque and 6.7% in atheromatous plaque respectively] were recorded only in people with healthy periodontium.

Discussion

Periodontal medicine deals with a two way relationship in which periodontal disease may powerfully influence on an individual's systemic health or disease or the more customarily understood role of systemic disease on influencing an individual's periodontal health or disease[3]. Moving away from the earlier studies linking periodontal disease with vascular disease, Pussinen and colleagues[6] reported that elevated antibodies to selected periodontal pathogens were associated with an increased prevalence of coronary heart disease, increased atherosclerosis in the carotid artery and more risk of developing coronary events during 10 years of follow-up. Beck and colleagues,[7] reported that increased systemic antibody titers to periodontal microbes were related to an increased prevalence of coronary heart disease. Recently, Spahr and Colleagues[8] provided evidence that these subclinical effects might be translated into clinical coronary disease. The available data suggests that the periodontal infections seem to be found more frequently in patients with CVD. There is, however, no direct peer-reviewed evidence to suggest that treating or preventing periodontal infections leads to fewer clinical cardiovascular events.

The present study was aimed to evaluate and compare the presence of targeted periodontal pathogens in subgingival plaque and atheromatous plaque samples from chronic periodontitis and periodontally healthy patients who had undergone endarterectomy procedure to treat their peripheral arterial disease. In present study demographic status included mean age group of 43.1 ±7.4yrs in Group A and 53.6±10.3 in Group B, which was statistically significant with p-value of < 0.05. The maximum incidence of PAD and chronic periodontitis is between 4th to 5th decades with peak incidence between 43-48yrs. That is why in group B mean age has shown 53.6±10.3 years which are more than the mean age of the patients in group A. The studies done by Grau et al 2004[9], Dietrich et al 2008,[10]Sim et al 2008[11] also found that 60yrs to 65yrs age group patients showed the stronger association when compared to younger individuals. Janket and co-workers 2003[12]also showed that relative risk of CVD was 1.44 which was higher in patients with periodontal diseases having age ≤65yrs.

The studies done by Grau et al. 2004[9], Sim et al. 2008[11] and Xu & Lu 2011[13] showed stronger association of periodontitis with CVD and cerebrovascular diseases in men than in women. On the contrary, Andriankaja et al. 2007[14] found a stronger association in women compared to men whereas Tuominen et al. 2003[15] found no association between periodontitis and coronary heart diseases in either sex.

A case control study done by Latronico et al[16] supported an epidemiological association between periodontitis and CHD, in which periodontal parameters such as deep pockets and number of missing teeth seems to be important risk factor for CVD. In the present study, in Group A, two patients (13.7%) had normal C reactive protein (CRP) values and thirteen (86.7%) had above normal values. In Group B, all patients had above normal CRP values. . In the present study, in Group A, two patients (13.7%) had normal C reactive protein (CRP) values and thirteen (86.7%) had above normal values. In Group B, all patients had above normal CRP values patients. Elevated CRP values in periodontally healthy patients might be because of PAD existing in patients in group A.

The same results were shown in studies done by Tuter G et al 2007 and Higashi Y et al 2008 and 2009[18] where they all have showed significantly higher serum high- sensitivity C-reactive protein concentrations in periodontitis patients with cardiovascular disease or hypertension than in patients without periodontitis. Fredriksson et al 1998[19], Losche W et al 2005[20],Blum A et al 2007[21] and Sun XJ et al 2009[13], Paraskevasn et al [22] demonstrated a positive association between periodontitis and C-reactive protein. Padilla et al[23].2006 was the first team who used a bacterial culture of homogenized samples, and then amplified the isolates with PCR technique.

Intergroup comparison was done for the detection of the periodontal pathogens, P.gingivalis, A.actinomycetemcomitans, P.intermedia and T. forsythia from subgingival plaques. Although the difference was not statistically significant, the results revealed that P.gingivalis was more prevalent in subgingival plaques of Group B(60%) compared to Group A(26.7%). The prevalence of A.a was more in Group A(26.7%) compared to Group B(20%). P.intermedia and T.forsythia were detectable (6.7% each) only in Group A and were not identified in Group B (graph 1& 2).

On analyzing the above results, subgingival plaque of Group A was found to harbor all the four target pathogens where as only two were present in group B. P.gingivalis and A. actinomycetemcomitans were detected in more number of plaque samples of patients in group B than in group A, this is probably because of increased subgingival bacterial load consisting more of these gram negative periodontal pathogens belonging to red complex in periodontal pockets as in the study of Kolenbrander et al. 2002[24]

Interestingly, A. actinomycetemcomitans often has been detected from periodontally healthy individuals Rylev&Kilian2008[25] but at lower levels than in subjects with periodontitis Hyvarinen et al. 2009.[26] This maybe the reason for its presence seen in periodontally healthy patients of Group A and P.intermedia and T.forsythia were not detected in the plaque sample of patients in group B and the reason might be insufficient quantity or improper method of collection plaque samples and smaller sample size.

Similarly intergroup comparison of the prevalence of target periodontal pathogens in atheromatous plaques of diseased peripheral arteries showed significantly more occurrence of P.gingivalis in Group B (46.7%) when compared to Group A (6.7%) with P value of 0.03. The prevalence of A.actinomycetemcomitans in atheromatous plaque was more in Group A (13.3%) as compared to Group B (6.7%).Zaremba et al 2007[26] in their study could identify P.gingivalis, A.actinomycetemcomitance, T.forsythia and C.retus in all the samples of atheromatous plaques collected from coronary artery, peripheral arteries like carotid, femoral, and abdominal aorta. Gaetti-Jardimet al 2009[27] observed 53.8%of P.gingivalis, 46.2% of A.actinomycetemcomitans in atheromatous plaque in patients with periodontitis.

In contrast to the results of our study, Aquinoet al 2011[28] observed 0% of P.gingivalis, Aimetti et al 2007[29] did not find any pathogen using Nested PCR. Romanoet al 2007[30] found 0% bacteria in atheromatous plaque using DNA probes, Padilla et al[23] 2006 did not identify any periodontal pathogen by culture and specific PCR. Marques da Silva et al 2005[31] also had similar results for P.gingivalis but the presence of A. actinomycetemcomitans was confirmed.

P.intermedia and T.forsythia were present in atheromatous plaques (13.3% each) only in group A and were not identified in group B. On the contrary, Figuero et al[32] observed 78.6% of P.gingivalis, 66.6% of A.actinomycetemcomitans and 60.1% of T.forsythia.

Intra-group comparison within group A (graph 3)showed the prevalence of P.gingivalis 6.7% in atheromatous plaque and 26.7% in subgingival plaque whereas prevalence of A.actinomycetemcomitans was 13.3 % in atheromatous plaque and 20% in subgingival plaque.

Prevalence of P.intermedia and T.forsythia was the same, 13.3% in atheromatous plaque and 6.7% in subgingival plaque. This reverse proportion of the occurrence of P.intermedia and T.forsythia observed in subgingival and atheromatous plaque in group A was because these pathogens were not identified in two subgingival plaque samples but were identified in atheromatous plaque samples.

Intra-group comparison within the group B (graph 4) showed prevalence of P. gingivalis 46.7% in atheromatous plaque and 60% in subgingival plaque showing statistical significance with P value of 0.001, whereas prevalence of A.a was 6.7% in atheromatous plaque and 20% in subgingival plaque showing significant difference with a P value of 0.04.P.intermedia and T.forsythia could not be identified in either of the plaque samples in group B. The presence of P.gingivalis and A.actinomycetemcomitans in the atheromatous plaque may be due to seeding of these bacteria from subgingival plaque through bacteremia and their translocation to the other part of the body like adventitia of peripheral arteries.[33] All the above mentioned studies have suggested the same possible mechanism in an attempt of explaining the possible reason of this positive association observed between chronic periodontitis patients and patients with CVD or/and PAD.

Conclusion :

Within the limitations of cross-sectional design with parameters recorded at one point of time and a relatively small sample size, our results provide the additional evidence to support the potential association present between chronic periodontitis and PAD. In chronic periodontitis patients, periodontal pathogens from subgingival plaque may gain the access to the systemic circulation to colonize at distant sites influencing the pathophysiology of atherogenesis.Prospective longitudinal studies and more interventional studies should be conducted in future to know the reality in the causality of the diseases.

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