Original Research Paper

Volume - 10 | Issue - 6 | June - 2020 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Dental Science

EFFECT OF NON-SURGICAL PERIODONTAL THERAPY ON CLINICAL PARAMETERS AND HUMAN CYTOMEGALOVIRUS (HCMV), EPSTEIN -BARR VIRUS (EBV) AND HERPES SIMPLEX VIRUS (HSV-1) IN CHRONIC PERIODONTITIS PATIENTS BY USING MULTIPLEX PCR METHOD – AN INTERVENTIONAL STUDY.

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ABSTRACT **INTRODUCTION:** Periodontitis is multifactorial, chronic disease. Before 1970s, periodontitis was believed to be caused by bacterial plaque. After evolution in microbiology, 8 herpes viruses infection identified in humans. Among these human herpes virus, studies reported the presence of cytomegalovirus, Epstein-barr virus and herpes simplex virus more frequently in periodontitis patients than others. The aim of this interventional study was to compare clinical parameters and to quantify the human herpes viruses like Human cytomegalovirus (HCMV), Epstein - Barr virus (EBV) & herpes simplex virus (HSV-1) in chronic periodontitis patients before and at 3 months following non-surgical periodontal therapy by using multiplex PCR method.

MATERIALS AND METHODS: This multiplex PCR method quantified the herpes viruses. Subgingival plaque samples were collected from 32 chronic periodontitis patients. Oral hygiene index - simplified, gingival index and probing depth were measured. Recording of clinical parameters and microbial analysis was carried out at baseline and at 3 months following the non-surgical periodontal therapy.

RESULTS: Statistically significant improvement found in all clinical parameters with statistically significant reduction in quantity of herpes viruses at 3 months following therapy.

CONCLUSION: This study demonstrated that treatment of chronic periodontitis by non-surgical periodontal therapy result in improvement in clinical parameters and reduction in quantity of herpes simplex virus – 1, Epstein – barr virus and human cytomegalovirus.

KEYWORDS : Chronic Periodontitis, human herpes virus, human cytomegalovirus, Epstein-barr virus, Herpes simplex virus-1, Multiplex PCR

INTRODUCTION

Periodontitis is a multifactorial, chronic disease that causes destruction of supporting structures of teeth - cementum, alveolar bone, periodontal ligament and gingiva.[1] Before 1970s, main etiology of periodontal disease was believed to be bacterial plaque but there was no any study that showed clear association between progressive periodontal disease and specific bacterial species.[2] In the 1970s, following the evolution of anerobic microbiology, many different micro-organisms were identified in supporting tissues of the teeth in periodontal disease.[3] Studies reported presence of A.a as primary etiologic factor in localized aggressive periodontitis and P.gingivalis in severe periodontitis.[4] This evolution has improved the treatment plan and since then it has become cause-related. In mid 1900s, presence of human herpes viruses were reported in progressive periodontal diseases.[5]

"Herepes" name come from greek word "Herpein" which means "to creep". From this nature of lesions, caused by the herpes viruses can be understood. Main function of herpes virus infections is immune impairment. 25 families are there in Herpetoviridae, but only 8 of them are known to infect humans. (Table 1).

Table-1 NAMES OF DIFFERENT TYPES OF HUMAN HERPES VIRUS

HHV – 1	Herpes simplex virus 1	HSV – 1
HHV – 2	Herpes simplex virus 2	HSV – 2
HHV- 3	Varicella zoster virus	VZV
HHV – 4	Epstein- Barr virus	EBV
HHV – 5	Cytomegalo virus	CMV
HHV – 6	Human Herpes Virus 6	HHV – 6
HHV – 7	Human Herpes virus 7	HHV – 7
HHV – 8	Kaposi's sarcoma- associated herpes virus	KSV

Specific types of the Herpes virus have been reported in progressive periodontal diseases such as Epstein barr, HCMV, and HSV-1. Herpes viruses exert cytopathic effect, which has direct effect on endothelial cells, inflammatory cells, fibroblasts and keratinocytes including PMNs, lymphocytes, macrophages, and also bone cells. In periodontitis, HHV-4 and HCMV infection showed to alter the activities of defence cells. Cytomegalovirus suppress cytotoxic T-lymphocyte functions, which decreases CD4+ cells and increases CD8+, which impairs cellular immunity. HHV-4 infected B lymphocytes, secretes antigens which produce blocking antibodies, immune complex formation, and activates T-suppressor cell.[7] Cytopathic effects of herpes virus may inhibit tissue repair and its turnover.[5and6]

Herpes virus infected Aggressive periodontitis contain fewer viable cells, more T & B lymphocytes than chronic periodontitis or healthy periodontium. Studies reported that sites with presence of herpes virus increase level of other microbiota, and mainly affect the periodontopathic bacteria, P. gingivalis, , Prevotella intermedia, Treponema denticola, *Tannerella* forsythia and A.a.[8] Herpes virus proteins on cell may act as receptors for bacteria. A.a. in periodontal pockets are also increased with EBV infection.[5]

Herpes viruses may induce abnormalities in the defence mechanism of PMNs, which are key defence cell against periodontal pathogen - bacteria. Herpes viruses exacerbate the disease, and co-infection of HCMV and Epstein - barr, or with HCMV and simplex virus, occur in different types of periodontal disease.[5]

Different diagnostic methods are available to identify viruses in perodontitis. Initially detection and examination of virus was carried out by culturing method.[9] Different techniques are available now such as, flow cytometry, DNA probes and immunofluorescene staining, to detect these viruses in periodontium.

Among these, Polymerase chain reaction is becoming the standard technique for detection and quantification of herpesvirus in periodontium.[10] Several types of PCR methods are used like Nested PCR, real time PCR, multiplex PCR.[11] Nested PCR techniques is more efficient in detecting low viral loads[18] while By multiplex PCR, multiple organisms can be detected at the same time.[19]

The purposes of this interventional study were to compare clinical parameters and to quantify the human herpes viruses like Human cytomegalovirus (HCMV), Epstein - Barr virus (EBV) & herpes simplex virus (HSV-1) in chronic periodontitis patients before and after non-surgical periodontal therapy at 3 months by using multiplex PCR method.

MATERIALS & METHODS

The Present study was conducted in the Department of Periodontology, K. M. Shah Dental College, Pipariya, Vadodara. 32

Chronic periodontitis patients were included from the OPD of department of periodontology, K M Shah Dental College and Hospital, SumandeepVidyapeeth (from January 2014 to July 2015). A total of 32 sample sites were used according to sample size calculation.(95% CI and 80% power. Formula: n = (z/ES)^2 = (2.802/0.5)^2 = 31.404 = 32). Recruitment of the participants and sites were done as per the inclusion and exclusion criteria. Patient diagnosed with chronic periodontitis, aged between 35 - 60 yrs, systemically healthy patients, with minimum of 20 teeth and ≥5mm probing depth were included. Site with deepest probing depth were used for sampling. Pregnant & lactating, habit of smoking & tobacco chewing. unwilling for participation in the study/ further follow up, any antimicrobial treatment in the previous 6 months, underwent periodontal therapy in the previous 6 months were excluded. The protocol was approved by Institutional Ethics Committee. Patients were explained about the full procedures and an informed consent form was obtained from all the participants.

Clinical examination were based on the following indices: Oral hygiene index simplified (OHI-S) (GREEN & VERMILION 1964), Plaque index (PI) (SILNESS AND LOE 1964), Gingival index (GI) (LOE AND SILNESS 1963), Bleeding on Probing (BOP) (Dichotomous 30sec), Probing depth (PD) All parameters were measured with UNC-15 periodontal probe and were performed on the six sites of each tooth (mesio-buccal, mid-buccal & disto-buccal and mesio-lingual, mid-lingual & disto-lingual)

SAMPLE COLLECTION

Plaque samples were collected from the site with ≥5mm probing depth in the entire dentition.. Prior to sampling, supragingival scaling was carried out of the tooth/site with ≥5mm probing depth. Site was properly isolated with cotton rolls and sterile paper points were then placed for 20 seconds in the site with deepest probing depth. (Figure-2) Immediately after collecting plaque, paper points were placed into the TE buffer – Transport media. (Figure-1 and 2). On the same day, collected plaque samples were sent for quantitative microbial analysis of HCMV, EBV and HSV-1 to Maratha Mandal's Nathajirao G. Helgekar Institute of Dental Science Department of Microbiology, Belgaum. Microbial examination of collected samples was done within 72 hours by Multiplex Polymerised Chain Reaction (PCR) technique. Clinical examination and microbial analysis were repeated at 3 months following scaling and root planning to evaluate its effect clinically and on quantity of human herpes viruses and before and at 3 months following scaling and root planning.

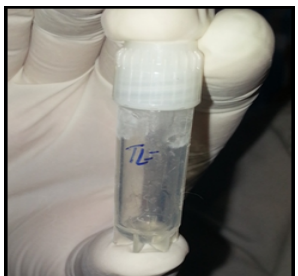

Figure-1 Te Buffer Solution As Transport Media

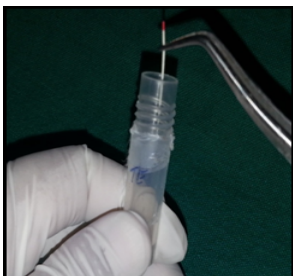

Figure-2 Collected Plaque Sample Stored In Te- Buffer Solution

Polymerase Chain Reaction(pcr) Procedure

Viral DNA extraction procedure was done by modified Proteinase – K method. Specific PCR primes were used for specific virus.

HSV-1 primer sequences
5'-CGT ACC TGC GGC TCG TGA AGT -3' as forward
5'-AGC AGG GTG CTC GTG TAT GGG C -3' as reverse
HCMV Primer sequences
5'-ACG TGT TAC TGG CGG AGT CG -3' as forward
5'-TTG AGT GTG GCC AGA CTG AG -3' as reverse
EBV Primer sequences
5'-AGC ACT GGC CAG CTC ATA TC -3' as forward
5'-TTG ACG TCA TGC CAA GGC AA -3' as reverse

A Qiagen Taq PCR Core Kit was used for polymerase chain reaction which contains following components, 10X CoralLoad PCR Buffer (Containing 15mM $MgCl_2$), dNTP mix 10mM of each, Taq DNA Polymerase 5 units/reaction. A thin walled PCR tube was placed on ice and the following components were added for each 20 µl reaction. First to get desired volume; calculated water was added to the tube, then buffer with remaining components were added. Thereafter it was mixed by pipetting up and down several times.

PCR was performed with a final volume of 20 µl mixture containing 5 pmol of each primer, 0.25 µl Taq DNA polymerase, Magnesium chloride, 1 µl Deoxynucleoside triphosphates (dNTP) mix and 2 µl of extracted DNA sample. These tubes were then placed in the thermal cycle and amplified using standard PCR procedure.

PCR was carried out under following conditions: The first step is DNA Denaturation step. The DNA was initially heated for 5 minutes at 95^{0}C and after that the Denaturation step was carried out for 30 sec at 95^{0}C for 45 cycles. The second step is Primer annealing step during which the PCR primer find their complementary targets and attach themselves to their sequences and it was done for 30 sec at 54^{0}C for 45 cycles. Finally, the last step in PCR cycle is polymerase extension step, during which the DNA polymerase produces a complementary copy of target DNA strand starting from PCR primer sequence. The polymerase extension step was carried out for 30 sec at 72^{0}C. Thereafter it was followed by the final extension period of 5 min at 72^{0}C and following PCR samples were kept at 4^{0}C. Amplified mix was subjected to electrophoretic sepration in Triethylenediaminetetraacetetic acid (EDTA) in 2% Agarose gel and viewed under transilluminator and gel documentation system as shown in figure – 3.

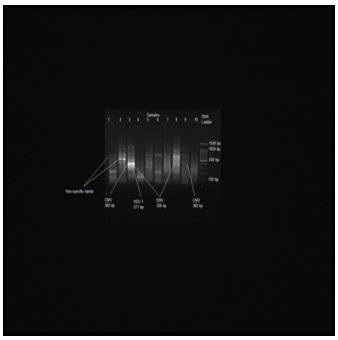

Figure-3 Multiplex Polymerase Chain Reaction

RESULTS

Paired t test was used for the comparison of clinical parameters at baseline and at 3 months following non-surgical periodontal therapy. Statistical comparisons of clinical parameters are shown in table -1. Significant difference was found between baseline and at 3 months following therapy (p value <0.001). Table – 2 shows that there was statistically significant decrease in quantity of herpes viruses following treatment.

Table -1Comparision Of Clinical Parameters At Baseline And After 3 Months By Paired T Test

CLINICAL PARAMETERS	PairedDifferences			
	Mean±Std. Deviation	Std. Error Mean	95%Confidence Intervalofthe Difference	p-value
ORALHYGEINE INDEX–SIMPLIFIED	2.750±0.803	0.142	3.040-2.0460	<0.001
PLAQUE INDEX	1.719±0.457	0.081	1.883–1.554	<0.001
GINGIVAL INDEX	1.562±0.504	0.089	1.744–1.381	<0.001
PROBING DEPTH	1.094±0.530	0.094	1.285–0.903	<0.001

Table-2 Comparison Of Microbiological Findings At Baseline And After 3months

	Median(IQR)	
	Baseline	3months
HSV-1	5550(34900)	2000(2725)
HCMV	7300(47900)	3300(2000)
EBV	2100(4800)	1000(600)

HSV – 1 Herpes simplex virus – 1, HCMV – Human Cytomegalovirus, EBV – Epstein – Barr virus

DISCUSSION

Present study was carried out to measure clinical parameters and quantity of human cytomegalovirus, Epstein-bar virus and herpes simplex virus-1 in chronic periodontitis patients and to evaluate the effect of non-surgical periodontal therapy on clinical parameters and on quantity of herpes viruses in periodontium. Very few studies have been done that showed effect of non-surgical periodontal therapy on herpes viruses and in the present study quantification of herpes viruses was carried out that showed statistical significant reduction at 3 months following non surgical periodontal therapy.

Studies reported that in the presence of little plaque, reactivation of these viruses may lead to periodontal destruction while stable condition of periodontium was observed in the presence of little plaque and periodontopathic bacteria with latent human herpes virus.[5] These studies reported that latent herpesviruses do not interfere in host response or aggravate colonization of periodontopathicbacteria. Also studies found positive correlation of active herpesviruses with the increase in the frequency of detection and counts for P. Gingivalis, P. Intermedia, A.a.[5,6,8] Presence of these both viruses and bacteria lead to progressive destruction of periodontium. As bacterial infection alone failed to explain all features of different types of periodontitis.[11]

Bacteria and herpes virus, their actions are bidirectional. Bacterial products or other inflammatory mediators have the potential to activate human herpesviruses.[5,12] In Experimental study on mice infected with cytomegalvirus and Porphyromonus Gingivalis showed a significantly higher mortality rate than mice infected with cytomegalovirus and E. coli.[12] P. gingivalis - bacteria has potential to suppress the antiviral host response that explains the enhancement of pathogenicity of HHV-5. Human herpes viruses and periodontal pathogenic bacteria, both have roles in progressive periodontal destruction. Balance between Pro- and anti-inflammatory mediators controlled by lymphocytes,that believe to be crucial in the pathogenesis of periodontal diseases. Elevated pro-inflammatory cytokines in periodontium are associated with periodontal destruction. The human herpes virus can inhibit the antibacterial host defence by inducing production of proinflammatory cytokines and chemokines, stimulate osteoclasts production, elevate MMP level, and decrease tissue inhibitors of metalloproteinase, this increases risk of tissue breakdown in periodontium by inhibiting tissue turnover rate and repair.[14]

In this present study, only chronic periodontitis patients were included, as studies reported positive association of herpes viruses in chronic and aggressive periodontitis patients than gingivitis patients and healthy individuals.[15-16] Also studies reported high prevalence of Epstein-barr virus in chronic and aggressive periodontitis patients compared to healthy individuals.[12]

Many studies reported detection of Epstein – bar and cytomegalovirus were found to be high in aggressive periodontitis than chronic periodontitis. Yaper M et al in 2003 studied on cytomegalovirus and Epstein – barr virus in aggressive periodontitis patients before and 3 months following the surgery. In this study, patients were underwent surgical procedure and antimicrobial therapy. Clinical and microbial examinations were carried out at baseline and after 3 months following the surgery. Statistically significant difference found in plaque index, gingival inflammation, attachment level and in presence of viruses after 3 months following the treatment.[15]

Another studies compared inflammatory cells in chronic and aggressive periodontitis patients before and after non-surgical periodontal therapy. These studies showed significant improvement in lymphocytes, immunoglobulin containing cells and plasma cells.[16-17]

Contreas and Slots in 2000 reported positive association of human cytomegalovirus, Epstein - barr virus and herpes simplex virus with bacterial infection and clinical severity of periodontium. They found these viruses significantly higher in deep pockets than shallow pockets.[12] Kubar et al reported presence of this herpes virus 80% in aggressive periodontitis and 46% in chronic periodontitis patients, which is statistically significant.[18]

CONCLUSION

Study showed significant effect of non surgical periodontal therapy on clinical parameters and quantity of herpes viruses in chronic periodontitis patients. Understanding the role of herpes viruses in periodontitis patients may become valuable in improving diagnosis, determining more specific treatment and in prevention of the disease progression.

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