Original Article

DNA Laddering to Evaluate Cytogenetic Damage in Patients with Periodontitis

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Abstract

Background: Inflammatory conditions show cytogenetic damage in peripheral blood leukocytes and this can be assessed using various tests. Cytogenetic damage as observed in the peripheral blood cells, is a marker of periodontal disease. DNA laddering is a sensitive assay which evaluates the cytogenetic damage. DNA laddering is a feature that can be observed when DNA fragments, resulting from apoptotic DNA fragmentation, are visualised after separation by gel electrophoresis which results in a characteristic "ladder" pattern. **Aim:** The aim of the present study is to investigate the cytogenetic damage in different forms of periodontitis in comparison with healthy controls. **Materials and Methods:** In this cross-sectional study, 15 systemically healthy subjects with moderate to severe chronic periodontitis (CGP), 15 systemically healthy subjects with generalised aggressive periodontitis(GAP) and 15 systemically healthy control subjects were recruited. Blood samples of the patients were drawn and evaluated for the cytogenetic damage by DNA laddering. **Results:** Apoptotic DNA fragmentation was observed as a "ladder" pattern at 180-200 BP intervals in both CGP and GAP groups indicating the DNA damage, in contrast with the healthy group where the ladder pattern was not observed suggesting of the healthy DNA. **Conclusion:** The results indicated that there are cytogenetic damages in both the chronic and aggressive periodontitis groups incontrast to the healthy controls.

Keywords: *Cytogenic damage, DNA laddering, periodontitis*

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Introduction

Periodontitis is a chronic inflammatory disease of tooth supporting tissues with conglomerate etiology of complex biofilm, behavioral factors, environmental, and genetic traits of the host.[1] According to Global Burden of Disease Study (GBD 1990–2010), periodontitis ranks the sixth most prevalent disease worldwide, with the global burden increased by 57.3% from 1990 to 2010.[2] Periodontitis is prevalent in at least 11% of people worldwide.[3]

Microorganisms are implicated as primary etiological agents. Although microbes have a established role in initiation of periodontal disease, the host immuno-inflammatory response to microbial onslaught plays a key role in periodontal disease progression and they are orchestrated by a number of host-related factors, either intrinsic or induced.[4]

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Chronic periodontitis (CGP) is characterized by slow progression of periodontal attachment loss, whereas generalized aggressive periodontitis (GAP) is characterized by rapid loss of periodontal tissues in otherwise clinically healthy subjects.[5]

Reactive oxygen species (ROS) have gained more attention, because of their central role to the progression of inflammatory diseases.[6] They are involved in normal cellular metabolism and continuously generated by the cells in most tissues. The role of ROS in the pathogenesis of periodontal disease has been illustrated by various mechanisms such as DNA damage, lipid peroxidation, protein disruption, and inflammatory cytokines, which contribute to the periodontal tissue destruction.[7-9] ROS can cause potential tissue damage by initiating free radical chain reactions.[10] Literature suggests that genetic damage has an important role to play in various chronic inflammatory and degenerative diseases such as Parkinson's disease,[11] chronic obstructive pulmonary disease (COPD),[12] inflammatory bowel disease,[13] Behcet's disease,[14] coronary artery disease,[15] and Raynaud's phenomenon.[16] The role of instability of the chromosomes and the propensity of these diseases has been evaluated with the help of various cytogenetic tests. Cytogenetic damage in peripheral blood leukocytes can be assessed using micronucleus test, flow cytometry, fluorescent assays, COMET assay, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay, and DNA laddering.[17] DNA laddering (fragmentation) was first described in 1980 by Andrew Wyllie at the University of Edinburgh Medical School. It is a sensitive assay that evaluates the cytogenetic damage that can be visualized as a ladder pattern of 180–200 base-pairs (bp) due to cleavage of the ds-DNA by the activation of endonucleases when run on a standard agarose gel electrophoresis.[18] The aim of this study was to evaluate the cytogenetic damage in chronic and GAP in comparison with healthy controls by using the DNA-laddering technique.

Materials and Methods

This study was cross-sectional in design, comprising of 45 patients who attended the Outpatient Department of Periodontics. A total of 74 patients were recruited for the study. Of these, 14 subjects dropped out, 15 did not meet the inclusion criteria, and finally 45 subjects completed the study. Informed consent was obtained from all the patients. Ethical clearance was obtained from the Institutional Ethical Committee and Review Board (Protocol no. SSCDS/2017/42). The sampling method is the non-probability sampling or convenience sampling where the subjects were recruited based on the following criteria [Figure 1].

Inclusion criteria

The inclusion criteria of the study included the following:

CGP group: This group comprised of 15 systemically healthy individuals with moderate-to-severe alveolar bone loss and attachment loss of 5 mm or more in multiple sites of all four quadrants of the mouth.

GAP group: This group comprised of 15 systemically healthy individuals with generalized pattern of severe destruction and attachment loss of at least 5 mm on at least three permanent teeth other than central incisors or first molars.

Control group: This group comprised of 15 systemically healthy subjects with no clinical evidence of periodontal disease who underwent oral prophylaxis before their treatment procedures such as restorations and orthodontia.

Exclusion criteria

The exclusion criteria of the study included any kind of systemic disease (hypertension, diabetes, etc.) that can change the course of periodontal disease, pregnancy/lactation, smokers or subjects consuming

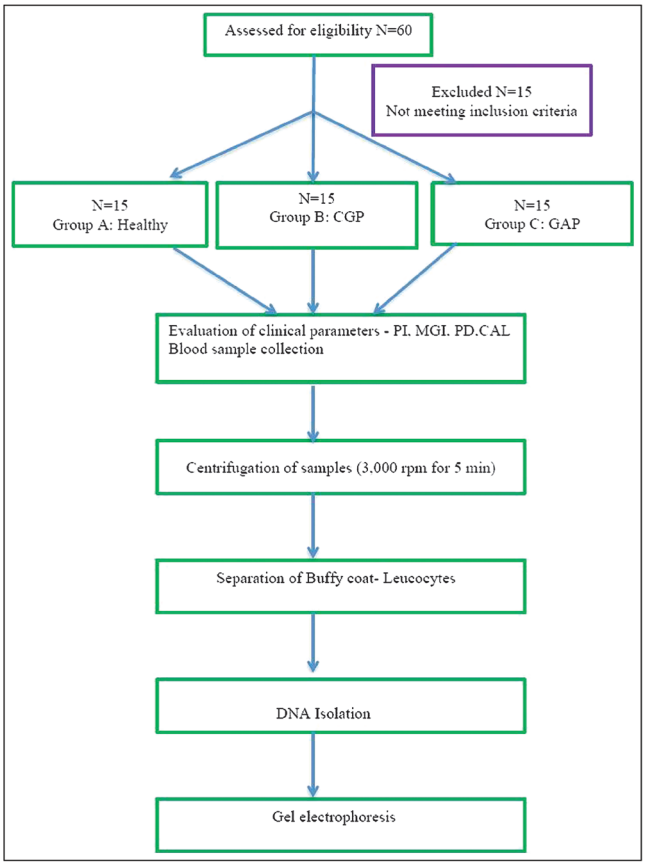

Figure 1: Consort diagram showing the patients allocation and the procedure

alcohol, subjects on antibiotics and anti-inflammatory medications within the past three months, history of any recent infections, and subjects with history of periodontal treatment in past six months prior to the study.

Procedure

After recruitment in the study, patients were subjected to a detailed general and clinical examination and the data pertaining to all the relevant parameters were recorded in a proforma specially designed for the study.

The clinical parameters included were probing pocket depth (PPD), clinical attachment level (CAL), modified gingival index (MGI), plaque index (PI), and bleeding index (BI).

For the blood collection, the skin over the antecubital fossa was disinfected and 5 mL of blood was collected by venipuncture using 20-gauge needle in a 5-mL syringe from each patient in all the three groups. Collected sample is centrifuged at 3,000 rpm for 5 min to separate white buffy coat that consists of leucocytes. A series of chemicals are added to lyze the cells and to dissolve RNA. The final isolated DNA is run on 2% agarose gel for electrophoresis to visualize the DNA fragmentation.

The grading of DNA fragmentation as mild (+), moderate (++), or severe (+++) can be categorized by the location of the bands that correspond to the fragmented DNA.

In the mild (+) degree, the location of the bands was nearer to the wells or in the upper third where the DNA sample was loaded. In the moderate (++) degree, the location of the bands was in the middle third, midway between the wells where the DNA sample was loaded and the end of the gel. However, in the severe (+++) degree, the location of the bands was away from the wells where the DNA sample was loaded and nearer to the end of the gel in the lower third.[19]

Results

All the clinical parameters in the patients with CGP and GAP were significantly higher ($P < 0.001$) than the control subjects. MGI and PI were significantly higher in the CGP and the GAP groups than the control group ($P < 0.001$). MGI was 1.71 ± 0.40 in Group B and 1.77 ± 0.26 in Group C. The mean PPD was 1.65 ± 0.15 mm in Group A, 6.57 ± 0.80 mm in Group B, and 6.74 ± 0.45 in Group C, respectively. The mean CAL was 5.89 ± 0.53 in Group B and 6.56 ± 0.24 in Group C [Table 1].

Apoptotic DNA fragmentation was observed as a 'ladder' pattern at 180–200 bp intervals in both CGP and GAP groups [Figure 2]. The gradation was 'moderate' as the location of the bands was in the middle third, midway between the wells where the DNA sample was loaded and the end of the gel [Table 2].

In this study, DNA fragmentation was observed in both CGP and GAP groups suggestive of the cytogenetic damage. DNA laddering was observed as a 'ladder' pattern at 180-bp intervals in the middle third of the wells in both the CGP and the GAP groups.

Discussion

Periodontitis, a chronic inflammatory disease, is caused by the periodontopathic bacteria such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, etc. Components of microbial plaque induce an initial infiltrate of inflammatory cells, including macrophages, polymorphonuclear leukocytes (PMNs), and lymphocytes. Microbial components, lipopolysaccharide (LPS), activate macrophages to synthesize and release a variety of proinflammatory molecules, cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha), prostaglandins, especially prostaglandin E2 (PGE2), and hydrolytic enzymes. These cytokines exhibit potent pro-inflammatory and catabolic activities and play a key role in periodontal tissue breakdown through production of collagenolytic enzymes such as metalloproteinases (MMPs). In the inflammatory environment, these latent collagenolytic enzymes are activated by ROS giving rise to increased levels of interstitial collagenases leading to periodontal breakdown. Evidence links the association of periodontitis with several systemic diseases such as diabetes, cardiovascular disease, COPD, adverse pregnancy outcomes, etc., where the periodontal inflammation directly contributes to systemic inflammation, through the release of toxins or leakage of microbial products into the bloodstream.[20] These chemical mediators cause destruction of extra cellular matrix components leading to an increase in ROS production, which is implicated in the connective tissue damage sustained during inflammatory diseases.

Table 1: Clinical parameters of patients of tests group and control group

Clinical parameters	Controls	CGP	GAP
Number of samples	15	15	15
Age (mean ± standard deviation [SD])	41.8 ± 5.83	45.2 ± 7.07	24.7 ± 3.63
MGI	0.00 ± 0.00	1.71 ± 0.40	1.77 ± 0.26
PI	0.20 ± 0.07	2.02 ± 0.30	1.86 ± 0.26
PD	1.65 ± 0.15	6.57 ± 0.80	6.74 ± 0.45
CAL	0.00 ± 0.00	5.89 ± 0.53	6.56 ± 0.24

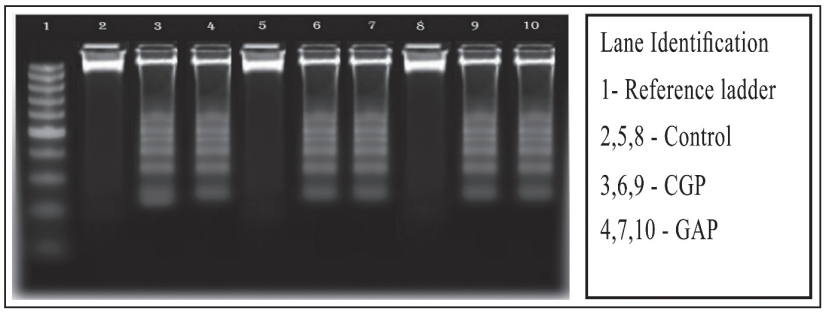

Figure 2: Agarose gel electrophoresis demonstrating the DNA fragmentation

Table 2: Grading of the samples of DNA ladder

Group	DNA ladder	Grading
A- (n = 15)	0(0%)	–
B-CGP (n = 15)	15(100%)	Moderate
C-GAP (n = 15)	15(100%)	Moderate

Neutrophil-mediated tissue injury was evaluated by Deguchi *et al.* [21] where they demonstrated that ROS causes substantial tissue damage by initiating free radical chain reactions. DNA damage can be evaluated by certain tests and methods among which DNA ladder is the most simple, sensitive, and cost-effective.[22]

DNA fragmentation assay using the agarose gel electrophoresis is a commonly-used technique for the detection of apoptosis.[23] It can easily discriminate between apoptotic and non-apoptotic (necrotic) modes of cell death. In most cases, the inter-nucleosomal cleavage of genomic ds-DNA by the endonucleases, like caspase-activated DNase (CAD), yields the characteristic DNA ladder, which is the molecular hallmark of apoptotic cells.[24,25] In the DNA ladder obtained, the pattern observed is that the molecular weights of the genome fragments are integer multiples of 180–200 bp length, which are associated with a nucleosome subunit. Separation of these fragments by agarose gel electrophoresis and subsequent visualization, for example, by ethidium bromide staining, results in a characteristic 'ladder' pattern [Figure 3]. In contrast, a DNA smear is obtained during agarose gel electrophoresis when genomic fragments of irregular sizes are induced during cell necrosis.[26]

The results indicated that there are cytogenetic damages in the periodontitis groups and this correlates with the literature that suggests that the DNA mutations and damages were observed in periodontitis group.[27-29] Numerous studies showed that the activity of producing ROS is higher in peripheral blood neutrophils of patients with periodontitis in contrast to healthy individuals.[30-32] There are studies suggesting a strong association between genetic polymorphism

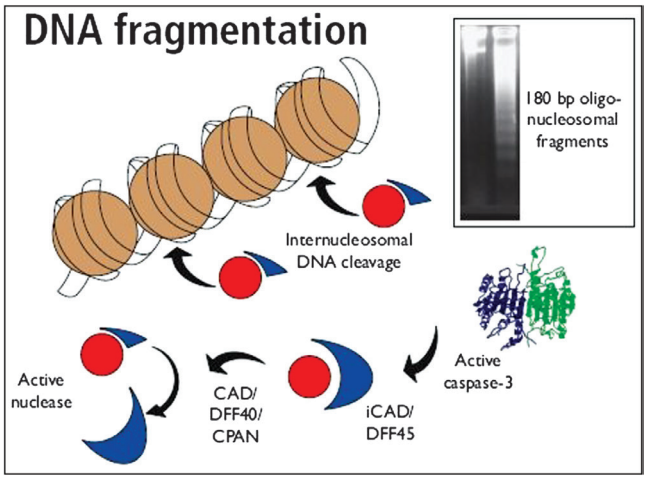

Figure 3: Diagrammatic representation of DNA fragmentation. (Source: https://static.bio-rad-antibodies.com/uploads/DNAFragmentationAntibodies.jpg)

and periodontitis.[33,34] These studies suggest that an increased ROS generation in periodontitis is not only due to stimulation by pathogens but also is genetically predisposed.[35] Cytogenetic damage was evaluated using a micronucleus test by Fenech *et al.*[36] where they found the cytogenetic damage in blood and in exfoliated buccal cells. The involvement of mitochondrial damage with the disease by the increased ROS production leading to decreased mitochondrial membrane potential and low oxygen consumption was put forward by Govindaraj *et al.* They also found that mtDNA mutations were observed in the gingival tissues but were absent in blood.[37] Damage of gingival tissues has led to mtDNA mutations in subjects with CGP and this was evaluated in a study by Canakci *et al.*[38] where they found 5-kb mtDNA deletion in the gingival tissues of the patients with CGP in Turkish population. A study evaluated positive correlation between oxidative stress produced by periodontitis disease and micronuclei. They reported that subjects with periodontitis (CP or AgP) showed an increase in the frequency of micronuclei that are directly related to DNA damage.[39] Another study evaluated oxidative stress-induced apoptosis

in H2O2-treated human periodontal ligament cells (hPDLCs) by TUNEL assay. They found significant reduction in viability and increased apoptosis in the hPDLCs which confirms the damage.[40]

In contrast, the findings of few studies did not relate the role of genetics and oxidative damage in the pathogenesis of periodontal disease. In a study by Haritha *et al.*, [41] no significant differences were found in the micronuclei frequency in the periodontitis groups as compared to the controls, thus indicating a possible lack of cytogenetic damage in peripheral blood cells. A few other studies also did not report a positive correlation between the cytogenetic damage and oxidative stress.[42,43]

Literature suggests that studies evaluated the effect of initial periodontal therapy on the oxidative stress markers that detects the DNA damage.[44,45] Studies reported that biomarkers of oxidative stress-induced DNA damage levels like 8-hydroxy-deoxyguanosine (8-OHdG) were significantly reduced by periodontal treatment.[46-49] However, no significant differences in the local levels of 8-OHdG between individuals with gingivitis and periodontitis,[50] as well as between patients with CGP and GAP [39] were observed. A study assessed the frequency of nuclear morphological changes in gingival epithelial cells by COMET assay in individuals with periodontitis, before and after periodontal therapy and compared the morphology to healthy periodontal tissues. They found that in periodontal disease the frequency of nuclear morphological changes increased in gingival epithelial cells and after the periodontal therapy there was a reduction of the number.[51]

Literature suggests the certitude of the DNA damage by the ROS released by chronic inflammatory conditions like metabolic diseases as well.[52,53] Therefore, to the best of our knowledge, this study is presumed to be the first of its kind to evaluate cytogenetic damage of ds-DNA of the nucleus in peripheral leucocytes using DNA laddering in periodontitis. Further studies with larger sample size are required to evaluate the genetic makeup and the predisposition of the ds-DNA for the correlation of the intra group variation between CGP and GAP groups and to assess DNA damage in the periodontal tissues by DNA-laddering technique.

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Conflicts of interest

There are no conflicts of interest.

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