

Effect of Low-dose Aspirin on Neutrophil Apoptosis in Periodontitis: An Immunohistochemical Study

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ABSTRACT

Aim and objective: A growing body of research today suggests that periodontitis is due to the failure of resolution pathways to restore homeostasis. Resolution is an active phenomenon of suppressing inflammation once its main objective of eliminating pathogens is achieved. Neutrophil apoptosis is an important step in promoting resolution. In chronic periodontitis, neutrophils linger for a long time in inflamed connective tissue and cause tissue destruction. Aspirin, a widely used anti-inflammatory and anti-thrombogenic drug, is known to cause neutrophil apoptosis by synthesizing aspirin-triggered lipoxin (15epiLXA4) a potent pro-resolving lipid mediator. This immunohistochemical investigation is designed to study the effect of low-dose aspirin therapy on neutrophil apoptosis in the gingival biopsies of patients suffering from periodontitis.

Materials and methods: The study population consisted of 30 periodontitis patients who were divided into two groups of 15 each as test group and control group. Test group patients were on low-dose aspirin (75 mg/150 mg) therapy for clinical management of class 1 and class 2 functional capacity cardiovascular disease. Periodontal parameters like plaque index (PI), bleeding index (BI), probing pocket depth (PPD), and clinical attachment loss (CAL) were recorded, gingival biopsies were obtained from all patients for quantitative analysis of neutrophil apoptosis by immunohistochemistry using p53 as a marker of apoptosis. Chi-square/Fisher's exact test was used to find the significance of study parameters on a categorical scale between two groups.

Results: Results indicated that there was a decrease in PPD and CAL and a marginal increase in neutrophil apoptosis in the test group in comparison with a control group.

Conclusion: Low-dose aspirin therapy may induce neutrophil apoptosis and improve PPD and CAL. Hence, it is presumed that it has the potential to be used as a host-modulating agent in the clinical management of periodontitis.

Clinical significance: The low-dose aspirin could be used as a host-modulating agent in the management of periodontitis.

Key messages: Increase apoptosis of neutrophils in the gingival biopsies of periodontitis patients who are on long-term low-dose aspirin therapy for CVD indicates that low-dose aspirin could be a potential host modality agent in the treatment of chronic periodontitis.

Keywords: Apoptosis, Aspirin, Neutrophil, Periodontitis.

World Journal of Dentistry (2021); 10.5005/jp-journals-10015-1879

INTRODUCTION

Periodontitis is an inflammatory disease that results from a complex interaction between plaque microorganisms and the host immune system.¹ These interactions lead to alteration in bone and connective tissue homeostasis causing local tissue destruction.² Resolution is a biological phenomenon of restoring tissue homeostasis.³ A growing body of research today suggests that chronic inflammatory periodontal disease is due to the failure of resolution pathways.⁴ Over the years, our knowledge of inflammation has matured and provided a better understanding of resolution. Resolution is an active phenomenon aimed at suppressing and extinguishing vibrant inflammatory reactions once its main objective of eliminating pathogens is attained.³ This involves a network of cells and biological mediators. Among the various cells involved in this process neutrophils play a critical role.⁴

PMNs are the most abundant cells of the innate immune system and form the first line of defenses against invading pathogens and play a prominent role in the initiation and progression of inflammatory response.⁵ The various defense mechanisms of neutrophils that destroy invading pathogens are also capable of inflicting damage to surrounding host tissues.⁶ There is ample evidence that neutrophil apoptosis and its clearance from the site of infection by macrophages is a major mechanism of promoting resolution.⁷ Delayed neutrophil apoptosis or/and impaired

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How to cite this article: Sachin S, Mohamed F, Kishore B, *et al.* Effect of Low-dose Aspirin on Neutrophil Apoptosis in Periodontitis: An Immunohistochemical Study. *World J Dent* 2021;12(6):441-445.

Source of support: Nil

Conflict of interest: None

clearance of apoptotic neutrophils contribute to the progression of chronic inflammation as in the case of chronic periodontitis.⁸

The term apoptosis was described by Kerr and associates in 1972, which is used to describe a morphologically distinct form of cell death.⁹ Apoptosis is an active and defined process of cell death, which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in the tissues during physiological and pathological conditions.¹⁰ Its molecular events include shrinkage of cells, internucleosomal chromatin fragmentation, the appearance of new membrane antigens, cross-linking of cell proteins, eventual disintegration of the cell by blebbing, and elimination of resulting fragments by scavenger phagocytes.¹¹

Aberrant regulation of apoptosis can contribute to a wide variety of human diseases such as neurodegenerative disease, cancer, rheumatoid arthritis, infectious diseases including AIDS, and several bacterial infections. Alteration of apoptosis is also seen in autoimmune diseases such as hepatitis and graft-vs-host disease. Decreased or inhibited apoptosis is a feature of many malignancies, autoimmune disorders, and viral infections.¹²

Apoptosis can be modulated by various stimuli including hormones, cytokines, growth factors, bacterial or viral infections, and immune responses. Among other factors, the products of two genes that encode proteins Bcl-2 and p53 have been shown to play a fundamental regulatory role in apoptosis. Bcl-2 is a member of a family of antiapoptotic proteins that can prevent or reduce cell death induced by a variety of stimuli. p53 is the protein product of a tumor-suppressor gene, and the expression of p53 can induce apoptosis. This protein is also implicated in the regulation of tissue dynamics and is specifically thought to induce apoptosis in terminally differentiated cells, including inflammatory cells.¹³

Aspirin is a widely used anti-inflammatory drug that suppresses inflammation by inhibiting cyclooxygenase pathways.¹⁴ It also promotes resolution by synthesis of 15-epi-lipoxin A4 (aspirin-triggered lipoxin).¹⁵ It is reported that 15-epi-LXA4 increases neutrophil apoptosis and facilitates their clearance from the site of infection leading to resolution and healing of the inflammatory lesion.¹⁶

Previous studies have shown that the patient who was on low-dose aspirin therapy for CVD have less periodontal attachment loss.^{17,18} This indicates that low-dose aspirin could be of therapeutic benefit for the treatment of chronic periodontitis. However, this hypothesis needs to be tested for a sound scientific evidence. Hence, the present investigation aims to study the effect of low-dose aspirin therapy on neutrophil apoptosis by quantitative analysis of apoptotic neutrophils in the periodontal tissue of patients suffering from periodontitis by immunohistochemistry.

MATERIALS AND METHODS

The study population consisted of 30 chronic periodontitis patients selected from the Department of Periodontology, MR Ambedkar Dental College, Bengaluru and Department of General Medicine, BR Ambedkar Medical College, Bengaluru. Ethical clearance was obtained from the Institutional Ethics Board of MR Ambedkar Dental College (MRADC&H/106/2009-10). Written informed consent was taken from the subjects who fulfilled the inclusion criteria of the study. Subjects were divided into two groups as study group and the control group. The study group consisted of 15 periodontitis patients who were on low-dose aspirin therapy (75 mg/150 mg) for class 1 and class 2 functional capacity cardiovascular disease. The

control group consisted of 15 systemically healthy periodontitis patients who were not on aspirin. The sample size was determined by the previous study conducted¹⁹ based on the SD of PI in the study group 0.18 and control group 0.35, mean difference 0.285, effect size 1.07, alpha error 5%, power 80%, for two-sided test sample required is 15 per group. This was calculated using nMaster software version 2.

Inclusion Criteria for Both Groups (Study and Control Group)

- Presence of bleeding on probing.
- Probing pocket depth ≥ 5 mm.
- Clinical attachment level ≥ 3 mm in a minimum of two sites.
- A minimum of 1 tooth needs extraction.

Inclusion Criteria for Study group

- Subjects who were on low-dose aspirin therapy (75 mg or 150 mg).

Exclusion Criteria

- Patients on any systemic antibiotics or other NSAIDs.
- Patients who were diabetic.
- Patients who smoked or used any other form of tobacco.
- Pregnant and lactating patients.
- Patients on hormonal replacement therapy.
- Patients who have undergone scaling or any periodontal treatment for the past 6 months.

Exclusion Criteria for Control Group

- Subjects who were on aspirin therapy (75 or 150 mg).

Periodontal parameters like plaque index (Silness and Loe), bleeding index (Ainamo and Bay), probing pocket depth, and clinical attachment loss (CAL) were recorded using a UNC 15 probe.

Collection of Specimen

The gingival biopsy specimens were harvested from both groups. The gingiva along with junctional epithelium was harvested using a 15 number BP blade from the tooth which was indicated for extraction. The biopsies were fixed in 4% buffered formalin and were transported to the Research Laboratory of Maratha Mandal's Nathajirao G Halgekar Institute of Dental Sciences, Belgaum where the specimens were embedded in paraffin wax and sectioned. The apoptosis of the neutrophils in the connective tissue was detected by immunohistochemistry by using p53 monoclonal mouse antihuman antibody as described by Jarnbring et al.²⁰ and were quantified.

Immunohistochemistry

The sections were fixed in 4% buffered formalin and embedded in paraffin section (3 μ m) cut and mounted on glass slides and air-dried at 56°C overnight, were dewaxed in xylene place in decreasing concentration of alcohol for rehydration. Antigen retrieval was carried out by immersing the slides in a whole buffer and incubating them in an enzyme retriever (Biogenex) for 95°C for 10 minutes. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline containing Tween [phosphate-buffered saline (PBS), 50 mm sodium phosphate pH 7.6, 200 mm NaCl, and 0.1% Tween 20] for 15 minutes, rinsed, and non-specific binding of IgG was blocked with 5% normal swine serum (NSS, lot 107, DAKO A/S) in PBS + Tween. Then, the primary

antibody p53 (1 µg/mL) was added and incubated at 20°C for 1 hour. The antibody was diluted in 5% NSS in PBS + Tween. The sections were rinsed again and overlaid with the secondary antibody for 45 minutes. The immunoperoxidase labeling was performed using the avidin-biotin complex (ABC). The sections were then incubated with the chromogen (3,3'-diaminobenzidine tetrahydrochloride 0.05%. DAKO A/S) and 0.001% hydrogen peroxide in PBS. The process was stopped with cold tap water and the sections were counterstained with Meyer's hematoxylin for 3 minutes and the sections dehydrated in a graded alcohol series, cleared in xylene, and mounted in Pertex.

Quantification of Apoptotic Neutrophils

The stained slides were screened and were examined by the observers using a light microscope (Leica DMRB) equipped with a digital video camera. Each section was analyzed for the p53 positive stained cells in the connective tissue. The area chosen for quantification was captured through the charge couple device (CCD) camera and transferred to the computer. Neutrophil apoptosis was quantified by manually counting neutrophils and estimating the percentage of p53 positively stained cells were quantified and graded by using a 0–3+ scale as described by Bulut et al.¹³ 0, no staining; 1+, stained cells comprising <10% of the cells; 2+, stained cells comprising 10–30% of the cells and 3+, stained cells comprising >30% of the cells. Three fields were selected from each slide and an average was taken and calculated. The calculations were performed at ×40 magnification. All slides were evaluated blindly by two independent observers and there were no interobserver errors.

Statistical Analysis

Descriptive statistical analysis was carried out. Results on continuous measurements are presented on the mean ± SD (Min–Max) and results on categorical measurements are presented in number (%). Significance is assessed at a 5% level of significance. Chi-square/Fisher's exact test has been used to find the significance of study parameters on a categorical scale between two groups.

RESULTS

The mean age of the study group was 54.4 ± 6.45 and that of the control was 44.4 ± 13.4 . The sex distribution is given in Table 1.

The mean plaque index among the test group was 1.28 ± 0.26 and that of the control group was 1.41 ± 0.35 , and there was no statistically significant difference between both the groups. The mean bleeding index among the study group was 82.45 ± 6.0 and that of the control group was 71.61 ± 7.2 , which showed a statistically significant difference between the two groups as shown in Table 2.

The mean probing pocket depth among the study group was 3.95 ± 0.68 and that of the control group was 4.58 ± 1.2 which showed no statistically significant difference. The mean clinical attachment loss in the study group was 5.52 ± 0.52 and that in the control group was 6.12 ± 0.81 which showed a statistically significant difference between the two groups as shown in Table 3.

Grade 0 neutrophil apoptosis among test and control was 6 and 8, respectively, grade I was 9 and 6, respectively, and grade II was 0 and 1, respectively, as shown in Table 4. No Grade III neutrophil apoptosis was noticed (Fig. 1).

Figure 2 depicts the neutrophil apoptosis among the study and control group.

Table 1: Sex distribution of the subjects

| | Male | Female | Total |
|---------------|-----------|------------|-----------|
| Test group | 4 (26.7%) | 11 (73.3%) | 15 (100%) |
| Control group | 5 (33.3%) | 10 (66.7%) | 15 (100%) |

Table 2: Mean comparison of periodontal parameters

| Clinical parameters | Study group | Control group | p value |
|---------------------|-----------------|-----------------|---------|
| Plaque index | 1.28 ± 0.26 | 1.41 ± 0.35 | 0.249 |
| Bleeding index | 82.45 ± 6.0 | 71.61 ± 7.2 | 0.0005* |

*Statistical significant

Table 3: Mean comparison of periodontal parameters

| Clinical parameters | Study group | Control group | p value |
|---------------------|-----------------|-----------------|---------|
| Pocket depth | 3.95 ± 0.68 | 4.58 ± 1.2 | 0.091 |
| CAL | 5.52 ± 0.52 | 6.12 ± 0.81 | 0.022* |

*Statistical significant

Table 4: Neutrophil apoptosis in both groups

| Neutrophil apoptosis | Study group | Control group |
|----------------------|-------------|---------------|
| Grade 0 | 5 (33.3%) | 9 (60%) |
| Grade I | 9 (60%) | 6 (40%) |
| Grade II | 1 (6.7%) | 00 |
| Total | 15 (100%) | 15 (100%) |

Chi-square = 2.743, $p = 0.2537$

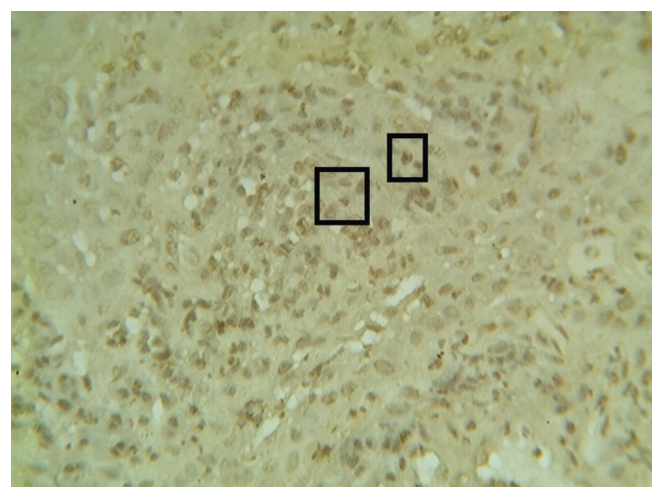


Fig. 1: Photomicrograph of gingival tissue stained with p53 monoclonal mouse antihuman antibody. Showing neutrophil apoptosis at magnification (×40)

DISCUSSION

Periodontal disease is a chronic inflammatory disease characterized by intricate interactions between pathogenic bacteria and the host defense system, which eventually leads to periodontal tissue destruction and tooth loss.²¹ Recent advances in the field of periodontal pathology have shown that the progression of periodontitis is due to the failure of resolution pathways in periodontal tissues.²² Resolution of inflammation is a coordinated and active process aimed at suppressing vibrant inflammatory

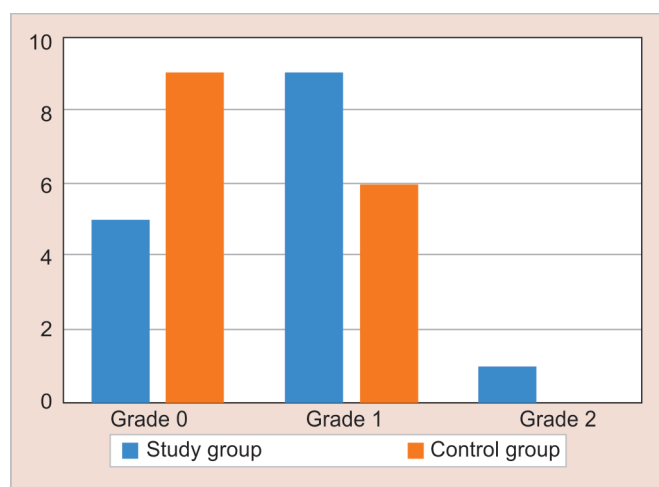


Fig. 2: Neutrophil apoptosis among study and control group

reaction and restoration of tissue integrity and function once its main objective of elimination of infection is attained.³ Neutrophils that form the first line of defense are the major cells of the innate immune system which play an important role in inflammation are also of critical importance in the process of resolution.²³ There is ample evidence to suggest that neutrophil apoptosis and their clearance by macrophages is an important step in initiation and promotion of resolution.⁷ Hence, it is intuitive to pharmaceutically modify the host responses to promote the resolution of inflammation.

Aspirin (acetylsalicylic acid) is a widely used non-steroidal anti-inflammatory drug. Its anti-inflammatory action is by suppression of pro-inflammatory prostaglandins synthesis by inhibition of cyclooxygenase (COX2) pathways.⁹ Recently, aspirin was found to cause a switch in eicosanoid biosynthesis as acetylation of COX2 changes the enzyme activity to produce 15-hydroxyeicosatetraenoic acid from arachidonic acid.²⁴ Human neutrophils use this substrate to produce a potent and stable pro resolving agent 15-epilipoxin A4 (15-epi LXA4) which is also called by the term aspirin-triggered lipoxin.²⁵

Aspirin-triggered lipoxin promotes apoptosis of neutrophils through attenuation of myeloperoxidase (MPO) triggered Mac-1-mediated survival signals.⁶ Aspirin, apart from its anti-inflammatory and pro-resolving action exhibits antiplatelet aggregating property which is mediated by suppression of thromboxane A2 synthesis. As a result, it is also used in cardiovascular patients.²⁶

p53 is a tumor suppressor protein called a guardian of the genome as it prevents mutation in the genome.²⁷ It is present in normal tissue and its expression can be detected with anti p53 antibodies using immunohistochemical techniques and it is used as a marker of apoptosis.¹³ This technique has been used for the detection of apoptotic neutrophils in the present study.

Dental plaque is the primary etiologic factor that initiates the periodontal disease. According to the non-specific plaque hypothesis, it is an increase in the quantity of plaque that plays a critical role in the pathogenesis of periodontitis. The present investigation showed that there was no difference in the plaque index between the test group and the control group. These findings are in accordance with the findings of Drouganis and Hirsch.¹⁷

Bleeding on probing is a clinical sign of periodontal disease and it increases with the severity of the disease. On contrary to this, in the

present investigation, the test group had a higher bleeding index in comparison with the control group. The difference is because of the anti-thrombolytic property of aspirin. These findings are in accordance with the previous studies.²⁸

An increase in probing pocket depth is a sign of periodontal disease and also an important clinical parameter that decides the choice of periodontal therapy. The present investigation revealed that the probing pocket depth in the test group was less compared to the control group. Similar findings were reported by previous studies.¹⁷ Clinical attachment loss is the most reliable clinical sign of tissue destruction in periodontitis. The present investigation showed that there was less clinical attachment loss among those patients who were on low-dose aspirin therapy than those who were not on aspirin therapy. This difference might be because of the anti-inflammatory and pro-resolving properties of aspirin and its derivative aspirin-triggered lipoxin. Similar results are reported by Faizuddin et al.¹⁸

Neutrophil apoptosis—a programmed cell death is a critical step in initiation and promotion of resolution which restores tissue homeostasis and helps in healing.²⁹ The present study revealed that the gingival connective tissue on chronic periodontitis patients had increased apoptotic neutrophils than those of the control group. Out of 15 samples, 9 showed grade-I, 1 showed grade-II, and 6 showed grade-0, whereas the control group showed 0 in grade-II, 6 in grade-I, and 8 in grade-0 revealing that the test group had more apoptotic neutrophils than the control group though the difference was not statistically significant. The difference might be due to the action of 15-epi-LXA₄ which tends to attenuate MPO triggered MAC-1-mediated survival signals thus promoting neutrophil apoptosis and help in the resolution of inflammation.

The overall findings of this investigation suggest that low-dose aspirin through 15-epi-LXA₄ promotes resolution of inflammation and restores periodontal tissue homeostasis and preserves periodontal health. However, this investigation has few limitations like advanced apoptosis detecting techniques are not used. And the patients in the test group are cardiovascular patients, no local debridement is carried out. Hence, conclusions about the efficacy of low-dose aspirin should be drawn with caution.

CONCLUSION

The results of this preliminary investigation reveal that there is an increase in the number of apoptotic neutrophils in the gingival biopsies of periodontitis patients who are on long-term low-dose aspirin therapy for CVD in comparison with biopsies of periodontitis patients who are not on aspirin therapy. This indicates that aspirin can increase apoptosis in inflamed tissues and could be a potential host modality agent in the treatment of chronic periodontitis. However, this hypothesis has to be further explored with well-designed RCTs in systemically healthy periodontitis patients before drawing final conclusions about the use of low-dose aspirin as an adjunct or an alternative to mechanical therapy.

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