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Research Article

The Effect of Nutritional Supplementation with Curcumin on RANKL/OPG Ratio in Gingival Crevicular Fluid of Chronic Periodontitis Patients: A Randomized Controlled Trial

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ABSTRACT

Background and Objectives: Although periodontitis is an infectious disease of the periodontium, changes that occur in bone are critical because the destruction of bone is responsible for tooth loss. A key system for controlling bone turnover is the RANK-RANKL-OPG system. Curcumin is a polyphenol with antioxidant, anti-inflammatory properties which has an inhibitory effect on NF κ ß. As an attempt to assess the effect of Curcumin on RANKL/OPG ratio levels in chronic periodontitis patients, this study was done to evaluate the effect of nutritional supplementation with curcumin on RANKL/OPG ratio in the gingival crevicular fluid of chronic periodontitis patients.

Methodology: The study was conducted in the Department of Periodontology, Rajarajeswari Dental College and Hospital. A total of 60 patients, Group I with 30 subjects, receiving a scaling and root planing and curcumin supplements in the form of tablets twice (500 mg) a day for 6 weeks. Group II with 30 subjects, receiving scaling and root planning and placebo twice a day for 6 weeks. All clinical parameters were recorded and GCF was collected from all the subjects at baseline and at 6 weeks after treatment to estimate the levels of RANKL/OPG ratio by ELISA.

Results: In the test group there was a significant decrease in a GI, PI, PD, CAL from baseline to 6 weeks. In both the groups there was a decrease in the RANKL/OPG ratio from baseline to 6 weeks after treatment but the decrease in the test group was more statistically significant.

Conclusion: Nutritional supplementation with curcumin showed a significant decrease in clinical parameters and also RANKL/OPG ratio at 6 weeks after treatment. Hence curcumin can be used as an adjunct in the treatment of periodontal diseases.

Keywords: Curcumin; ELISA; GCF; Periodontitis; RANKL/OPG

INTRODUCTION

Periodontitis is infectious diseases of the periodontium, changes that occur in bone are critical because the destruction of bone is responsible for tooth loss. The height and density of the alveolar bone are normally maintained by equilibrium, regulated by local and systemic influences, between bone proliferation and bone resorption. When resorption exceeds formation, both bone height and bone density may be reduced.

The factors involved in bone destruction in periodontal disease are bacterial and host-mediated. Bacterial plaque products induce the differentiation of bone progenitor cells into osteoclasts and stimulate gingival cells to release mediators that have the same effect [1]. Plaque products and inflammatory mediators can also act directly on osteoblasts or their progenitors, inhibiting their action and reducing their numbers. Several host factors released by inflammatory cells are capable of inducing bone resorption *in vitro* and play a role in periodontal disease. These include host-produced prostaglandins and their

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precursors, Interleukin-1 α (IL-1 α), Interleukin-1 β (IL- β), and Tumor Necrosis Factor-alpha (TNF- α) [2].

Biomarkers Definitions Working Group' biomarker is defined as 'A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. 'Research into biomarkers of periodontitis is driven by mainly three targets: to identify 'at risk' patients before periodontal tissue destruction occurs, to determine disease activity and progression; and to build up our understanding of this complex disease with the purpose of finding new therapeutic targets.

Whilst blood, saliva and Gingival Crevicular Fluid (GCF) has been particularly attracted as a marker of the periodontal disease activity because it contains elevated levels of a vast array of biochemical factors, proteins, and peptides which are possible markers for periodontitis and other diseases [3]. Hence the study on GCF samples is used as a marker for periodontal disease activity.

A key system for controlling bone turnover is the Receptor Activator of Nuclear Factor-Kappa ß (RANK)/Receptor Activator of Nuclear Factor-Kappa ß Ligand (RANKL)/ Osteoprotegerin (OPG) system. RANK is a cell surface receptor expressed by osteoclast progenitor cells as well as mature osteoclasts. RANKL is a ligand that binds to RANK and is expressed by bone marrow stromal cells, osteoblasts, and fibroblasts. Binding of RANKL to RANK results in osteoclast differentiation and activation and thus bone resorption. Another ligand that binds to RANKL is OPG, produced by bone marrow stromal cells, osteoblasts, and Periodontal Ligament (PDL) fibroblasts. Thus RANKL and OPG are cytokines that bind to RANK, resulting in cellular responses. However, although RANKL promotes activation and differentiation of osteoclasts, OPG has the opposite effect, inhibiting differentiation of osteoclasts. The balance between OPG and RANKL activity can, therefore, drive bone resorption or bone formation [4].

IL-1 β and TNF- α regulate the expression of RANKL and OPG, and T-cells express RANKL, which binds directly to RANK on the surfaces of osteoclast progenitors and osteoclasts, resulting in cell activation and differentiation to form mature osteoclasts [5]. Resulting in cell activation and differentiation to form mature osteoclasts. In periodontitis, elevated levels of proinflammatory cytokines, such as IL-1 β and TNF- α, and increasing numbers of infiltrating T-cells result in the activation of osteoclasts via RANK, resulting in alveolar bone loss. It has been reported that levels of RANKL are higher and levels of OPG are lower in sites with active periodontal breakdown compared to sites with healthy gingiva, and in GCF, RANKL/OPG ratios are higher in periodontitis than health [6]. It is clear that alterations in the relative levels of these key regulators of osteoclasts play a key role in the bone loss that characterizes the periodontal disease.

The investigational product is curcumin reconstituted with turmeric oil and dispensed in tablets (Curcubest). Curcumin is dispensed as tablets (500 mg), consumed twice daily orally after food for 6 weeks. Placebo consists of a gelatin capsule containing starch which is identical in physical characteristics and dispensed similar to the curcumin. The curcumin and placebo will be procured from Vin Super Foods, India. Curcubest with FSSAI-11215302000126 is an herbal product with no known side effects.

To the best of our knowledge not many articles in the literature that assess and compare the effect of nutritional supplementation with curcumin on the RANKL/OPG ratio in the gingival crevicular fluid of chronic periodontitis patients.

Hence this study was carried out to evaluate the effect of nutritional supplementation with curcumin on the RANKL/OPG ratio in the gingival crevicular fluid of chronic periodontitis patients among the South Indian population.

AIMS AND OBJECTIVES

The study was carried out to evaluate the effect of nutritional supplementation with curcumin on the RANKL/OPG ratio in the gingival crevicular fluid of chronic periodontitis patients among the South Indian population.

Objectives of the study

- To evaluate and estimate the effect of nutritional supplementation with curcumin on the RANKL/OPG ratio in the gingival crevicular fluid of chronic periodontitis patients
- To study the use of curcumin as an adjunct in the treatment of periodontal disease by the following parameters

Chronic periodontitis patients who are systemically healthy with probing pocket depth of ≥ 5 mm and clinical attachment loss ≥ 3 mm in patients aged between 30-60 years with radiographic evidence of bone loss.

METHODOLOGY

This is a randomized clinical controlled trial conducted to study the therapeutic effect of nutritional supplementation with curcumin on the RANKL/OPG ratio in the gingival crevicular fluid of patients with chronic periodontitis and also to study the use of curcumin as an adjunct in the treatment of periodontal diseases.

Patient selection

A total of 60 patients (age range: 30-60 years) were randomly allocated by using a random number table method from the Department of Periodontology, Rajarajeswari Dental College and Hospital, Bangalore. Subjects were explained about the study and based on their approval; written informed consent was taken after being advised about the nature of the study according to a protocol approved by the Ethical Committee of the Rajarajeswari Dental College and Hospital, Bangalore. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The subjects were distributed into two groups:

Group I (Test group): 30 subjects, who will receive scaling and root planing and curcumin supplements in the form of tablets twice (500 mg) a day for 6 weeks.

Group II (Control group): 30 subjects, who will receive scaling and root planing and placebo twice a day for 6 weeks. Placebo consists of a gelatin capsule containing starch.

A tablet consumption monitoring checklist with color-coded medicine dispensing boxes was given to the patient's family member to ensure better patient compliance. Volumetric micropipettes will be placed extra crevicular at the entrance of gingival crevice, and the GCF samples (2 × 3 µl) will be collected from each patient at baseline and at 6 weeks after treatment for both groups (Figure 1).

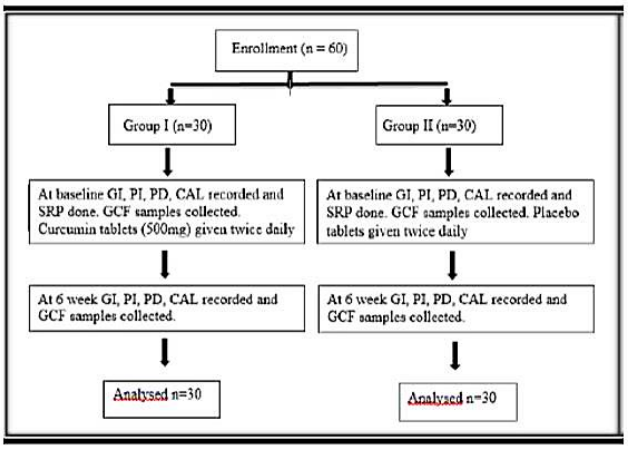

Figure 1: Flow chart of study groups.

Inclusion criteria

- Patients suffering from chronic periodontitis
- Patients between the ages group 30-60 years
- Probing pocket depth ≥ 5 mm
- Clinical attachment loss ≥ 3 mm
- Number of remaining teeth ≥ 20 excluding third molars
- Systemically healthy patients
- Patients with no history of allergy to drugs used or prescribed in this study

Exclusion criteria

- History of underlying systemic disease
- Pregnant and lactating females
- Patients who have undergone periodontal treatment within a period of 6 months
- Under antibiotic/NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) treatment during the past 6 months
- Patients with plaque index >1 and who don't follow oral hygiene instructions after Phase I therapy
- Patients on steroid therapy and oral contraceptives
- History of tobacco chewing and current or former smoker
- Patients who did not accept the terms and conditions of the study

Clinical periodontal examination

The following parameters were assessed at six sites per tooth

- Gingival Index (GI) (Loe et al.) [6]
- Plaque Index (PI)
- Probing Depth (PD)
- Clinical Attachment Level (CAL)

RANKL ELISA procedure

Principle of the assay: The RayBio® Human RANKL ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human RANKL in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for human RANKL coated on a 96-well plate. Standards and samples are pipetted into the wells and RANKL present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated antihuman RANKL antibody is added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of RANKL bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Kit components: RANKL Microplate (Item A)-96 wells (12 strips × 8 wells) coated with anti- Human RANKL.

- Wash Buffer Concentrate (20X) (Item B)-25 ml of 20X concentrated solution
- Standard Protein (Item C)-2 vials of Human RANKL
- Detection Antibody RANKL (Item F)-2 vials of biotinylated anti-Human RANKL
- HRP-Streptavidin Concentrate (Item G)-200 µl 500X concentrated HRP-conjugated streptavidin
- TMB One-Step Substrate Reagent (Item H)-12 ml of 3, 3, 5, 5'-tetramethylbenzidine (TMB) in buffer solution
- Stop Solution (Item I)-8 ml of 0.2 M sulfuric acid
- Assay Diluent A (Item L)-30 ml of diluent buffer
- Assay Diluent B (Item E)-15 ml of 5X concentrated buffer

Additional materials used:

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 2 µl to 1 ml volumes
- Adjustable 1-25 ml pipettes for reagent preparation
- 100 ml and 1 litre graduated cylinders
- Absorbent paper
- Distilled or deionized water
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard or sample dilutions

Reagent preparation:

- Bring all reagents and samples to room temperature (18℃-25℃) before use
- Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water before use

- Sample dilution: Assay Diluent C (Item L) should be used for dilution of serum, plasma, and cell culture supernatant samples. The suggested dilution for normal serum/plasma is 2 fold
- Preparation of standard: Briefly spin a vial of Item C. Add 400 µl Assay Diluent C (Item L) into Item C vial to prepare a 100 ng/ml standard solution. Dissolve the powder thoroughly by a gentle mix. Pipette 300 µl Assay Diluent C into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent C serves as the zero standards (0 ng/ml)
- If the Wash Concentrate (20X) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer
- Briefly spin the Detection Antibody vial (Item F) before use. Add 100 µl of 1X Assay Diluent B (Item E) into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4 ℃ for 5 days). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B (Item E) and used in step 5 of Part VI Assay Procedure
- Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent B (Item E)

For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 50 µl of HRP-Streptavidin concentrate into a tube with 10 ml 1X Assay Diluent B to prepare a 200-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix Well.

Assay procedure:

- Bring all reagents and samples to room temperature (18 ℃-25 ℃) before use. It is recommended that all standards and samples be run at least in duplicate
- Label removable 8-well strips as appropriate for your experiment
- Add 100 µl of each standard (see Reagent Preparation step 3) and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature with gentle shaking
- Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or auto washer. Complete removal of the liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels
- Add 100 µl of 1X prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking
- Discard the solution. Repeat the wash as in step 4
- Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking
- Discard the solution. Repeat the wash as in step 4
- Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking
- Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately

Statistical analysis

The study data will be analysed using SPSS (Statistical Package for Social Sciences) v.22 (IBM, Corp.,) for Windows [7].

Descriptive statistics: Descriptive analysis of all the explanatory and outcome parameters will be done using mean and standard deviation for quantitative variables, frequency, and proportions for categorical variables.

Inferential statistics:

- Independent student paired T-test is used to compare the mean scores of PI, GI, and PPD at baseline between the two study groups. Mann Whitney U test is used to compare the mean scores of biomarkers i.e. RANKL, OPG and RANKL/OPG ratio at baseline between the two study groups
- Student paired T-test is used to compare the mean scores of PI, GI, PPD at baseline and postoperative 6 weeks period within each study groups
- Wilcoxon signed-rank test is used to compare the mean scores of biomarkers i.e. RANKL, OPG and RANKL/OPG ratio at baseline and postoperative 6 weeks period in each group.
- Spearman's Correlation test is used to assess the relationship between clinical parameters and biomarkers at Baseline and 6 weeks period

  The level of significance will be set at $p<0.05$

RESULTS

A total of sixty patients were included in the present study. They were divided into two groups:

Group I (Test group): 30 subjects, who will receive scaling and root planning and curcumin supplements in the form of tablets twice (500 mg) a day for 6 weeks.

Group II (Control group): 30 subjects, who will receive scaling and root planning and placebo twice a day for 6 weeks. Placebo consists of a gelatine capsule containing starch.

All the patients completed the study; there are no dropouts cross verified with checklist forms and with color-coded medicine dispensing boxes (from amazon). None of the patient's complaint of any discomfort during/after sample collection and during the course of the study. All measurements were made at baseline and 6 weeks after treatment. The mean levels of RANKL and OPG were estimated in the GCF. There was no statistically significant difference in the age and gender

distribution among the test group with respect to the control group.

Demographic variables

Age: Estimation of the mean age changes in both the groups was assessed by using the Mann Whitney U test. The age of participants ranged between 37-56 years in Group I (Test group) with a mean age of 46.3 ± 5.9, 37-55 years in Group II (Control group) with a mean age of 44.8 ± 4.8.

Gender: Estimation of gender changes between both groups was assessed using the Chi-square test. There were 17 males and 13 females in Group I (Test group), 12 males and 18 females in Group II (Control group).

Periodontal variables

Gingival index: The intergroup comparison of mean values of the Gingival Index (GI) at baseline was assessed by using the Independent student T-test. At baseline, the mean GI in the control group and test group is 2.10 ± 0.28 and 2.03 ± 0.32 respectively, which did not show statistically significant differences (Table 1).

Table 1: Comparison of mean values of clinical parameters between test and Control groups at baseline period using the independent student T-test.

Parameters	Baseline		6 weeks		Mean diff	t	p-value
	Mean	SD	Mean	SD			
GI	2.03	0.3	2.1	0.3	-0.07	-0.88	0.38
PI	1.66	0.5	1.56	0.4	0.1	0.866	0.39
PD	7.74	0.9	7.4	0.8	0.35	1.621	0.11
CAL	3.27	0.8	3.39	0.7	-0.13	-0.65	0.52

Table 2: Comparison of mean values of clinical parameters between baseline and 6 weeks period in the test group using student paired T-test.

Parameters	Baseline		6 weeks		Mean diff	t	p-value
	Mean	SD	Mean	SD			
GI	2.03	0	1.11	0	0.91	15.6	<0.001*
PI	1.66	1	0.77	0	0.89	9.61	<0.001*
PD	7.74	1	7.43	1	0.31	9.64	<0.001*
CAL	3.27	1	3.12	1	0.15	6.17	<0.001*

* - Statistically Significant

The intragroup comparison of mean values of GI between baseline and 6 weeks after treatment in the test group was assessed using the Student Paired T-test.

With respect to baseline at 6 weeks in the test group, the mean value of GI significantly decreased from 2.03 ± 0.32 to GI is 1.11 ± 0.38 respectively (p<0.001) (Table 2).

The intragroup comparison of mean values of GI between test and control groups at 6 weeks after treatment was assessed using the Independent Student T-test.

At 6 weeks the GI showed a statistically significant difference (p<0.001) in the test group (1.11 ± 0.38) with respect to the control group (1.67 ± 0.28) (Figure 2).

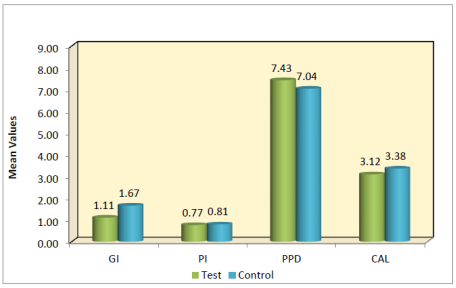

Figure 2: Comparison of mean values of clinical parameters between test and control groups at 6 weeks post-intervention.

Plaque index: The intergroup comparison of mean values of the Plaque Index (PI) at baseline was assessed by using the Independent student T-test. At baseline, the mean PI in the control group and test group is 1.56 ± 0.38 and 1.66 ± 0.51 respectively, which did not show statistically significant differences (Table 1).

The intragroup comparison of mean values of PI between baseline and 6 weeks after treatment in the test group was assessed using the Student Paired T-test.

With respect to baseline at 6 weeks in the test group, the mean value of PI significantly decreased from 1.66 ± 0.51 to 0.77 ± 0.11 respectively (p<0.001) (Table 2 and Figure 3).

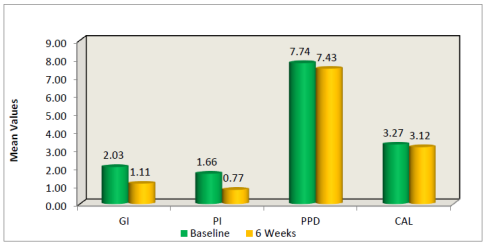

Figure 3: Comparison of mean values of clinical parameters between Baseline and 6 weeks post intervention period in test group.

The intragroup comparison of mean values of PI between Test and Control groups at 6 weeks after treatment was assessed using the Independent Student T-test. At 6 weeks the PI did not show any statistically significant difference between the test group (0.77 ± 0.11) and the control group (0.81 ± 0.10) (Figure 2).

Probing depth: The intergroup comparison of mean values of the Probing Depth (PD) at baseline was assessed by using the Independent student T-test. At baseline, the mean PD in the control group and test group is 7.40 ± 0.81 and 7.74 ± 0.85

respectively, which did not show statistically PD between baseline and 6 weeks after treatment in the test group were assessed using Student paired T-test.

Compared to baseline at 6 weeks in the test group, the mean value of PD found to be statistically significantly decreased from 7.74 ± 0.85 to 7.43 ± 0.84 respectively (p<0.001) (Table 2 and Figure 3).

The intragroup comparison of mean values of PD between Test and Control groups at 6 weeks after treatment was assessed using the Independent Student T-test. At 6 weeks the PD did not show any statistically significant difference between the test group (7.43 ± 0.84) and the control group (7.04 ± 0.80) (Figure 2).

Clinical attachment level: The intergroup comparison of mean values of the Clinical Attachment Level (CAL) at baseline was assessed by using the Independent student T-test. At baseline, the mean CAL in the control group and test group is 3.39 ± 0.68 and 3.27 ± 0.83 respectively, which did not show statistically significant differences (Table 1).

The intragroup comparison of mean values of CAL between baseline and 6 weeks after treatment in the test group was assessed using the Student Paired T-test.

Compared to baseline at 6 weeks in the test group, the mean value of CAL significantly decreased from 3.27 ± 0.83 to 3.12 ± 0.79 respectively (p<0.001) (Table 2 and Figure 3). The intragroup comparison of mean values of CAL between Test and Control groups at 6 weeks after treatment was assessed using the Independent Student T-test.

At 6 weeks the CAL did not show any statistically significant difference between the test group (3.12 ± 0.79) and the control group (3.38 ± 0.73) (Figure 2).

Biomarkers

Receptor Activator of Nuclear Factor Kappa β Ligand (RANKL): The intergroup comparison of mean values of the RANKL at baseline was assessed by using Mann Whitney Test.

Table 3: Comparison of mean values of biomarkers between test and control groups at baseline period using Mann Whitney test.

Parameters	Baseline		6 weeks		Mean Diff	Z	p-value
	Mean	SD	Mean	SD			
RANKL	254.07	54.5	236.01	47.14	18.06	-1.39	0.17
OPG	90.97	38.42	86.64	31.43	4.33	-0.651	0.52
RANKL/ OPG	3.12	1.07	3.04	1.14	0.08	-0.429	0.67

At baseline, the mean RANKL in the control group and test group is 254.07 ± 54.50 and 236.01 ± 47.14 respectively, which did not show statistically significant differences (Table 3).The intragroup comparison of mean values of RANKL between baseline and 6 weeks after treatment in test Group was assessed using the Wilcoxon Signed-Rank test. Compared to baseline at 6 weeks in the test group, the mean value of RANKL significantly decreased from 254.07 ± 54.50 to 109.10 ± 28 respectively (p<0.001) (Table 4 and Figure 4).

Table 4: Comparison of mean values of biomarkers between Baseline and 6 weeks period in test group using the Wilcoxon Signed Rank test.

Parameters	Baseline		6 weeks		Mean diff	Z	p-value
	Mean	SD	Mean	SD			
RANKL	254.07	54.5	109.1	28	144.97	-4.782	<0.001*
OPG	90.97	38.42	280.14	95.05	189.18	-4.782	<0.001*
RANKL/ OPG	3.12	1.07	0.42	0.15	2.7	-4.782	<0.001*

*: Statistically Significant

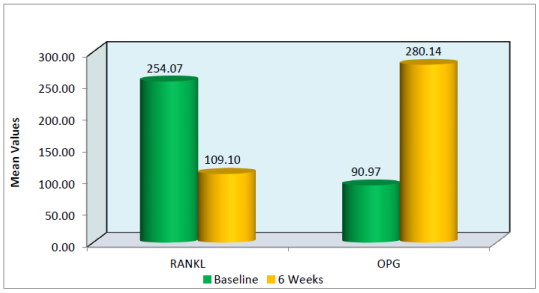

Figure 4: Comparison of mean values of biomarkers between baseline and 6 weeks post-intervention period in test group.

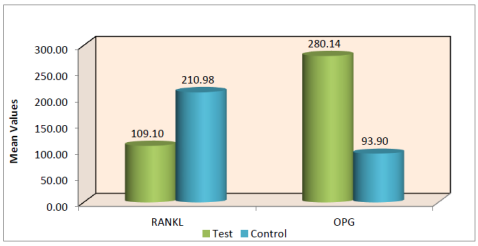

Figure 5: Comparison of mean values of biomarkers between test and control groups at 6 weeks post-intervention period.

The intragroup comparison of mean values of RANKL between Test and Control groups at 6 weeks after treatment was assessed using the Mann Whitney Test. With respect to the control group at 6 weeks in the test group, the mean value of RANKL significantly decreased from 210.98 ± 43.48 to 109.10 ± 28.0 respectively (p<0.001) (Figure 5).

Osteoprotegerin (OPG): The intergroup comparison of mean values of the OPG at baseline was assessed by using Mann Whitney Test. At baseline, the mean OPG in the control group

and test group is 254.07 ± 54.50 and 236.01 ± 47.14 respectively, which did not show statistically significant differences (Table 3). The intragroup comparison of mean values of OPG between baseline and 6 weeks after treatment in test Group was assessed using Wilcoxon Signed Rank Test. Compared to baseline at 6 weeks in the test group, the mean value of OPG significantly increased from 90.97 ± 38.42 to 280.14 ± 95.05 respectively ($p<0.001$) (Table 4 and Figure 4). The intragroup comparison of mean values of OPG between Test and Control groups at 6 weeks after treatment was assessed using the Mann Whitney Test. With respect to the control group at 6 weeks in the test group, the mean value of OPG significantly increased from 93.90 ± 31.02 to 280.14 ± 95.05 respectively ($p<0.001$) (Figure 5).

RANKL/OPG ratio: The intergroup comparison of mean values of the RANKL/OPG ratio at baseline was assessed by using Mann Whitney Test. At baseline, the mean RANKL/OPG ratio in the test group and control group is 3.12 ± 1.07 and 3.04 ± 1.14 respectively, which did not show statistically significant differences (Table 3).

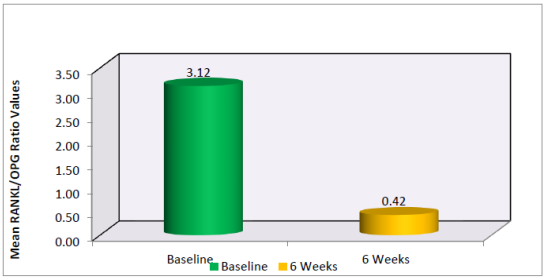

Figure 6: Comparison of mean RANKL/OPG ratio between baseline and 6 weeks post intervention period in test group.

Table 5: Spearman's correlation test to assess the relationship between clinical parameters and biomarkers at Baseline and 6 weeks period.

Group	Clinical parameters	Baseline		6 weeks	
		rho	p-value	rho	p-value
Test	GI	-0.1	0.64	0.25	0.19
	PI	-0	0.92	0.1	0.61
	PD	-0	0.96	0.01	0.98
	CAL	0.01	1	0.32	0.08
Control	GI	-0.1	0.53	0.01	1
	PI	-0.1	0.46	0.06	0.76
	PD	-0.2	0.26	-0.2	0.22
	CAL	0.04	0.83	0.19	0.31

The intragroup comparison of mean values of the RANKL/OPG ratio between baseline and 6 weeks after treatment in the test group was assessed using the Wilcoxon Signed-Rank test. Compared to baseline at 6 weeks in the test group, the mean value of the RANKL/OPG ratio significantly decreased from 3.12 ± 1.07 to 0.42 ± 0.15 respectively ($p<0.001$) after treatment (Table 4 and Figure 6). The intragroup comparison of mean values of the RANKL/OPG ratio between Test and Control groups at 6 weeks after treatment were assessed using the Mann Whitney Test. With respect to the control group at 6 weeks in the test group, the mean value of the RANKL/OPG ratio significantly decreased from 2.48 ± 0.91 to 0.42 ± 0.15 respectively ($p<0.001$) (Figure 7).

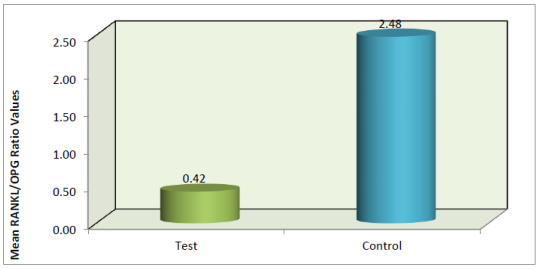

Figure 7: Comparison of mean values of RANKL/OPG ratio between test and control groups at 6 weeks post-intervention period.

Correlation to assess the relationship between clinical parameters and biomarkers: The correlation to assess the relationship between clinical parameters and biomarkers at baseline and 6 weeks period is done using spearman's correlation test. With respect to the control group in the test group, we found that PI and PD have a very strong correlation with respect to the biomarkers *viz* RANKL/OPG ratio (Table 5). It is observed that GI and CAL are found to have a very weak correlation with respect to the biomarkers *viz* RANKL/OPG ratio (Table 5).

DISCUSSION

Recent research has focused on the role of biomarkers to assess the current disease status and to predict the risk and outcome of the treatment provided. Various molecules including inflammatory mediators, host-derived enzymes, leucocyte break down products, and acute-phase proteins have been identified in oral fluids like saliva and GCF. Along with various inflammatory mediators that can be assessed in periodontitis RANKL/OPG ratio is one of the biomarkers that can be measured in inflammatory conditions to assess the bone status [8].

The role of cytokines in periodontal disease has been evaluated in several studies [9-12]. Recent studies have delineated many of the molecules, especially cytokines, required for osteoclast formation. In particular, osteoclast formation from precursor cells, as well as osteoclast activation, requires the receptor activator of NF κ ß ligand.

Osteoclasts are multinucleated giant cells that originate from hematopoietic stem cells of the macrophage/monocyte lineage. The differentiation of macrophages into osteoclasts requires the presence of Bone Marrow Stromal Cells (BMSC) [7,13,14] or osteoblast progeny. These accessory cells produce essential factors for osteoclastogenesis. Osteoclastogenesis is dependent upon exposure to Receptor Activator of Nuclear Factor- κ B Ligand (RANKL), a transmembrane glycoprotein expressed on

the surface of stromal cells in bone [15]. Osteoclast precursor cells that express the Receptor of Activator of Nuclear Factor-κB (RANK), interact with BMSCs and differentiate into mature osteoclasts upon stimulation by RANKL. RANKL interacts with RANK and leads to the recruitment of TNFR-Associated Factors (TRAF) [15]. Sequential recruitment of TRAF6 and NF-κB inducing kinase by RANK results in Nuclear Factor (NF)-κB activation, and recruitment of TRAF2 causes c-Jun N-terminal kinase activation, which promotes osteoclastogenesis [16,17]. Agents that suppress RANKL signaling reduce the bone loss induced by elevated osteoclastogenesis [15].

A study by Bharati A-C et al. have demonstrated that RANKL induces NF-κß activation through activation of IkB kinase (IKK) and IkB phosphorylation and degradation, and curcumin inhibits RANKL-induced NF-κß activation and osteoclastogenesis. Osteoprotegerin (OPG), a secreted glycoprotein, is a decoy receptor for RANKL [18]. When OPG is present to bind to RANKL, the cell-to-cell signaling between marrow stromal cells and osteoclast precursors is inhibited, and osteoclasts are not formed [18,19]. Thus, RANKL and 80 decoy receptors OPG expressed by bone-associated cells play important roles during osteoclast formation by balancing induction and inhibition [1].

Curcumin found to have anti-oxidative and anti-inflammatory properties and thereby inhibit tumor cell growth. It also inhibits transcription factors such as NF-κB and AP-1, down-regulates cyclooxygenase-2, nitric oxide synthase, matrix metalloproteinase-9, TNF, and chemokines, which are considered to be pro-inflammatory molecules [1]. It also inhibits osteoclastogenesis by blocking NF-κB activation in Raw 264.7 cells [1]. Cell studies *in vitro* and animal studies suggest that curcumin also affects osteoclast activity and inhibits bone resorption by suppression of osteoclastogenesis [14]. It has been proposed that curcumin inhibits NF-κß (transcription factor nuclear factor-kappa ß) and its ligand RANKL (Receptor activator of Nuclear Factor-Kappa ß Ligand) [20]. NF-κß is involved in impaired bone formation in osteoporosis and also inhibits the differentiation and mineralization of mature osteoblasts. Inhibiting NF-κß results in stimulating the differentiation and mineralization of primary murine bone marrow stromal cells and pre-osteoblasts [2,21]. Thus, curcumin through its interaction with NF-κß can enhance osteogenesis.

In a study by Oh S et al., [15] demonstrated that curcumin reduces the expression of RANKL in response to IL-1α in BMSCs by studying the effect of curcumin on levels of RANKL and OPG mRNA in BMSCs. They also stated that the inhibitory effect of curcumin on IL-1α-induced osteoclastogenesis was caused by inhibition of RANKL expression [15]. In a study by Ozaki K et al., [19] Curcumin has been shown to induce apoptosis in osteoclasts. It is possible that the apoptotic effects of curcumin are responsible for the suppression of osteoclastogenesis [22]. In a study by Zambrano LMG et al., [5] demonstrated that local application of curcumin in nanoparticle vehicle in the LPS-induced model of experimental periodontal disease inhibits inflammatory bone resorption, which is associated with a reduction in osteoclast numbers and inflammation [23].

In the present study, a total of sixty patients were included. Patients were divided into two groups Group I (Test group) with 30 subjects, who will receive scaling and root planing and curcumin supplements in the form of tablets twice (500 mg) a day for 6 weeks. Group II (Control group) with 30 subjects, who will receive scaling and root planing and placebo twice a day for 6 weeks. Placebo consists of a gelatin capsule containing starch [24].

The present study investigated the GCF levels of RANKL/OPG ratio in chronic periodontitis patients and the results has revealed that the mean RANKL/OPG ratio in test group who received scaling and root planing along with nutritional supplementation with curcumin tablets (500 mg) at baseline was 3.12 ± 1.07 pg/ml and after 6 weeks follow up was 0.42 ± 0.15 pg/ml ($p<0.001$). In the control group who received scaling and root planing along with placebo the mean RANKL/OPG at baseline was 3.04 ± 1.14 pg/ml and after 6 weeks follow up was 2.48 ± 0.91 pg/ml.

CONCLUSION

As an attempt to assess the effect of Curcumin on RANKL/OPG ratio levels in chronic periodontitis patients it is concluded that the levels of RANKL/OPG in chronic periodontitis patients significantly decreased after nutritional supplementation with curcumin for a period of 6 weeks. The present study indicated that the measurement of the RANKL/OPG ratio in GCF as a potential biomarker of bone resorption activity of periodontal disease presence. Curcumin because of its anti-inflammatory and wound healing properties showed a significant decrease in GI, PI, PD and CAL at 6 weeks after treatment. Curcumin also showed a significant decrease in RANKL/OPG ratio because of its inhibitory effect on NF κß (anti-inflammatory property).

Hence it is concluded that Curcumin can be effectively used as an adjunct in the treatment of periodontal diseases and also examining RANKL/OPG ratio levels in GCF might help in identifying patients with a periodontal breakdown or those who are at risk for periodontal inflammation.

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