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Clinical Evaluation of the Effects of Probiotics on Salivary Nitric Oxide Levels in Chronic Gingivitis Subjects during Orthodontic Treatment: A Clinico - Biochemical Study

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Abstract **Original Research Article**

Aim: To evaluate and compare salivary levels of nitric oxide in chronic gingivitis patients undergoing orthodontic treatment. ***Objective:*** To clinically evaluate the efficacy of probiotics on salivary levels of nitric oxide in chronic gingivitis patients undergoing orthodontic treatment. ***Materials and Methods:*** Unstimulated whole salivary samples were collected from a total of 20 subjects with chronic gingivitis undergoing fixed orthodontic treatment. Patients were divided into 2 groups. Group I patients were treated with non-surgical periodontal therapy i.e. Scaling and root planning (SRP) alone and Group II patients were treated with non-surgical periodontal therapy i.e. Scaling and root planning (SRP) and a Lactobacillus reuteri containing probiotic supplement. The clinical parameters of gingivitis were assessed using Modified Gingival Index (MGI), Sulcus Bleeding Index (SBI) and Plaque Index (PI). The biochemical analysis of salivary nitric oxide levels of subjects was conducted at baseline and 21 days. Statistical analysis was done using Mann-Whitney Test. ***Results:*** Group II patients showed a significant difference ($p<0.05$) as compared to Group I patients in terms of Salivary Nitric Oxide levels, Modified Gingival Index (MGI), Sulcus Bleeding Index (SBI) and Plaque Index (PI). The use of probiotics in combination with SRP in the treatment of chronic gingivitis during active orthodontic treatment phase showed better results when compared to SRP alone. ***Conclusion****:* Salivary Nitric Oxide proves to be a significant marker of gingival inflammation and the adjunctive use probiotics along with NSPT can be recommended to reduce the severity of gingival inflammation and plaque formation during ongoing orthodontic treatment phase.

Keywords: Nitric oxide, inflammation, saliva, gingivitis, orthodontic treatment, dental plaque, scaling and root planning (SRP), non- surgical periodontal therapy (NSPT), probiotics, lactobacillus reuteri.

INTRODUCTION

Periodontal disease is a plaque biofilm induced chronic inflammatory disease characterized by pathological manifestation of the host response against specific bacterial challenge that leads to progressive destruction of the tooth supporting periodontal tissues gingiva. It results when the equilibrium between the microbiota and the host response is disrupted. Pathogenicity of the microbiota, dental plaque biofilm, suppression of commensal bacteria and/or reduced host response are the key factors in the development of inflammatory periodontal disease. Specifically, dental plaque at the gingival tissue and tooth interface provides a favourable niche for the growth of bacteria and their simultaneous protection from antimicrobial agents and host defences. Bacterial plaque retention for a longer duration can thus lead to gingival inflammation [1].

The presence of fixed orthodontic treatment appliances such as the orthodontic bands on the surface of teeth cause difficulty in efficient removal dental plaque due to inadequate accessibility for oral hygiene. The impaired plaque removal and the mechanical biofilm traps by the fixed appliance promotes continuous accumulation of plaque and retention for microbial growth. The retention of bacterial plaque for a longer duration causes irritation of the gingival tissue that can lead to inflammation of gingiva. This may further cause gingivitis in patients who do not institute proper oral hygiene measures and can become an established lesion in 21 days [2].

The treatment for gingival inflammation aims to eliminate the etiologic factors which encompasses mechanical and chemical plaque control and patient education, non-surgical periodontal therapy (NSPT) which comprises scaling and root planning (SRP) and adjunctive antimicrobial therapy [3].

Nitric oxide (NO) production in the form of reactive oxygen species has been recognised as a marker of inflammation. Nitric oxide is a free radical gas which acts as a physiological and pathophysiological mediator in the biological system. It is an ubiquitous intercellular messenger molecule with important neuronal and immune functions [4]. Nitric oxide has been linked to the etiopathogenesis of periodontal disease [5]. Inducible nitric oxide synthase (iNOS) is an enzyme expressed by salivary glands and salivary nitrite concentration is used as biomarker of human exposure to nitrate from dietary sources. The salivary levels of NO of patients with gingivitis have been reported to be higher than those of healthy individuals. It has been reported that the pharmacological inhibition of NO or its actions can therapeutically be valuable in the disease management. The levels of nitric oxide can determine the severity and the state of the underlying disease process [6].

Probiotics are bacterial strains usually isolated from human commensal microbiota and adequately characterized for strain identity, content, stability with proven health effects. The most commonly used species of probiotics belong to the Lactobacillus, Bifidobacterium, Escherichia, Enterococcus and Bacillus genera. Probiotics are important for immune system development and regulation, maintenance of a healthy lining of the gastrointestinal tract, food digestion, synthesis of amino acids (nitrates), proteins and different vitamins, absorption of calcium, iron and vitamin D and have antibacterial effect. In periodontal disease, studies have investigated the role of probiotics in gingivitis and reported a significant decrease in terms of plaque and gingival indices, bleeding on probing and gingival inflammation in the probiotic groups. The use of probiotics in the treatment of gingivitis can therefore be postulated to be beneficial [7].

Hence, the present study was aimed to evaluate and compare the effects of use of probiotics containing lactobacillus reuteri on salivary levels of nitric oxide in subjects with chronic gingivitis undergoing orthodontic treatment.

MATERIALS AND METHODS

Ethics Statement

All the study subjects gave a written informed consent was prior to the start of the study. The study protocol was approved by Institutional Research Ethics Committee registered under Indian Council of Medical Research (ICMR) and Approval number YCDC/IECIRC /2021-2022/203.

Study Design and Selection of patients

The present study was an interventional parallel design single-blind randomized clinical trial for an experimental period of 21 days. The study was conducted in the Department of Periodontology, Department of Orthodontics and Department of Biochemistry in the respective dental college and hospital. The study aimed to evaluate the efficacy of probiotics on salivary levels of nitric oxide in chronic gingivitis subjects undergoing orthodontic treatment.

***Inclusion criteria*:**

1. Subjects within the age group of 13-33 years
2. Subjects undergoing orthodontic treatment with chronic gingivitis
3. Subjects without any systemic or salivary gland disease
4. Subjects who have not been previously undergone scaling and root planning during the orthodontic treatment phase.
5. Subjects who are reliable in their response to carry out the test measurements.

***Exclusion criteria*:**

1. Subjects who had taken any antibiotic or anti-inflammatory drug in last 6 months.
2. Subjects having any adverse habits such as smoking or tobacco chewing.
3. Pregnant and lactating mothers.

A total of 20 Subjects were selected for the study. They were randomly allocated to 2 groups of 10 subjects each. A detailed case history was recorded for each subject. Recording of clinical parameters for assessing chronic gingivitis and the collection of saliva was done at Baseline for GROUP I and GROUP II subjects before performing the scaling and root planning (SRP) and at 21 days after performing the scaling and root planning (SRP) for GROUP I subjects & SRP and a probiotic supplement to GROUP II subjects. The subjects in the Group II were adviced to take probiotic capsule (Pyloflush, Lupin Pvt. Ltd, India) containing 10 billion cells of lactobacillus reuteri each and once/daily half hour before meal.

Clinical Parameters

Clinical parameters for gingival inflammation were recorded using mouth mirror and William's graduated periodontal probe and a plaque disclosing solution (Plak-Check,Vishal Dentocare, India).

1. Modified Gingival Index (Lobene, Weatherford, Ross, Lamm and Menaker 1986) [8].
2. Sulcus Bleeding Index (Muhlemann H.R and Son.S 1971) [8].
3. Plaque Index (Turesky – Gilmore- Glickman Modification of The Quigley – Hein 1970) [8].

Saliva collection and Biochemical estimation of Salivary Nitric Oxide (NO) [9-12]

Samples of 4 ml of unstimulated saliva were collected in a plain sterile vacutainer (plastic test tube with a rubber stopper for vacuum seal) using a micropipette. The samples were then transferred to laboratory for a biochemical analysis of NO. The estimation of NO was done by assessing the nitrite levels in the saliva which a stable end product of nitric oxide metabolism. 0.5 mL of 1 N Sodium hydroxide (CAS No.1310-73-2, Modern Industries, Nashik) was added as stabilizer in the salivary samples to maintain the nitrite levels. The deproteinization was of salivary samples was done using 0.2 mL of 0.5M zinc sulphate ($ZnSO_4$) aliquot of 4 mL saliva and this mixture was then used for centrifugation. Centrifugation of salivary samples at 3000 rpm for 15 mins was done and the obtained supernatant was utilized. Salivary estimation of NO was carried out using the Griess colorimetric reaction. The Griess reagent (SRL, Sisco Research Laboratories Pvt. Ltd) solution (1:1 mixture of 1% sulphanilamide and 0.1% N-naphthyl ethylene diamine dihydrochloride in 5% orthophosphoric acid) reacted with solution of standard nitrite present in saliva to produce a purple coloured azo dye end product, the intensity of this colour is directly proportional to the concentration of NO present in the salivary sample which was measured on photo-electric colorimeter. A standard curve was obtained using the readings given by the colorimeter at different concentrations of the standard solution of sodium nitrite (Qualigens, Thermo Fischer Scientific India Pvt. Ltd) and a graph of optical density (absorbance) versus the concentration of nitrite was plotted. The graph showed linear relationship of optical density (salivary NO level) with concentration of standard sodium nitrite. The correlation of the optical densities to the concentration of nitrite was done using the standard nitrite curve and salivary levels of nitric oxide in the form of sodium nitrite were evaluated for each patient in Group I and Group II.

Figure 1 Armamentarium used in the study

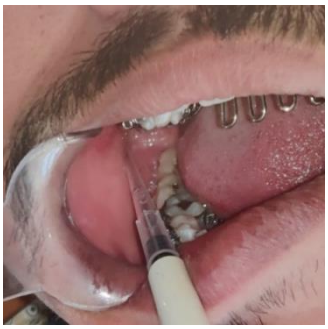


Figure 2: Salivary sample collection by Micropipette

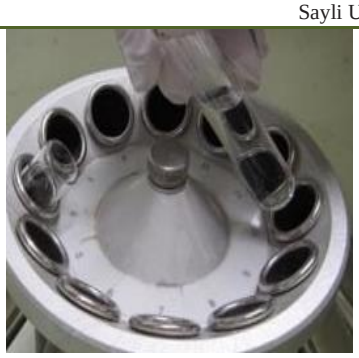


Figure 3: Centrifugation of salivary sample

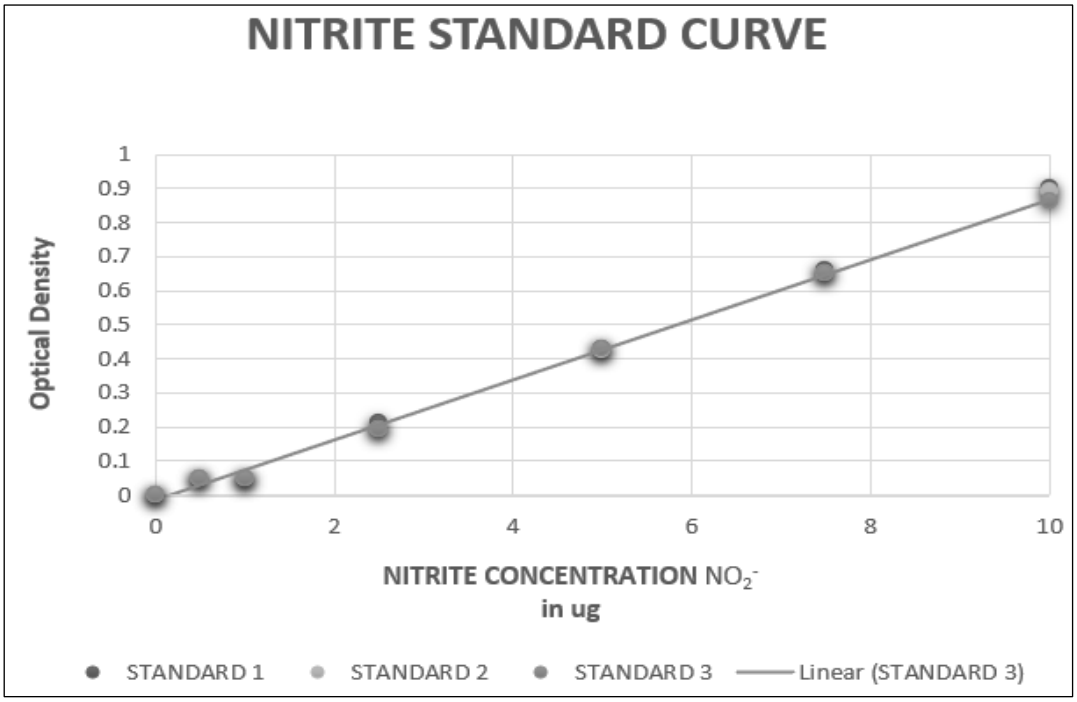


Figure 4: Nitrite Standard Curve Preparation

Figure 5: Preparation of Standard Nitrite Solutions at different concentrations

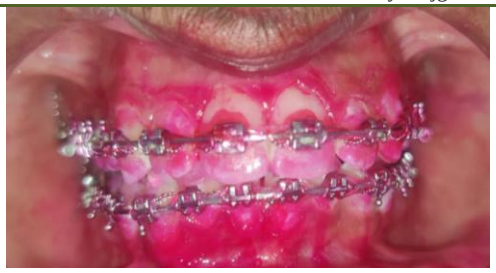


Figure 6: GROUP I at Baseline

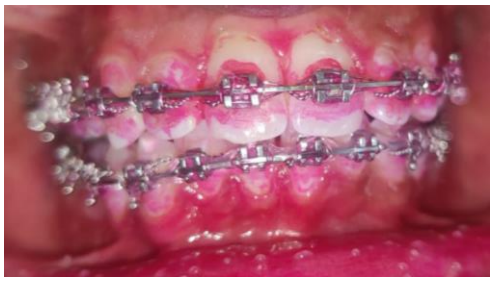


Figure 7: GROUP II at 21 Days

Figure 8: Salivary samples prepared for colorimetric analysis of Nitric Oxide using Griess Reagent

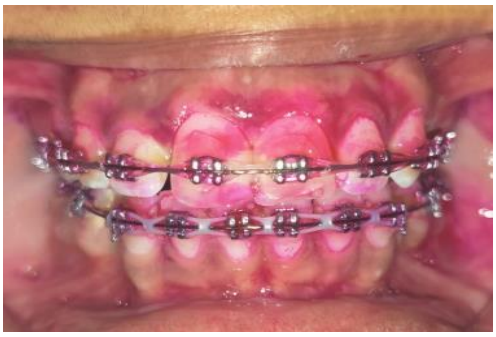


Figure 9: GROUP II at Baseline

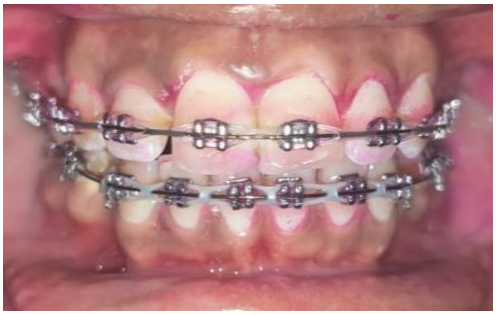


Figure 10: GROUP II at 21 Days

Figure 11: Probiotic Supplement

Figure 12: Colorimetric analysis of Salivary Nitric oxide

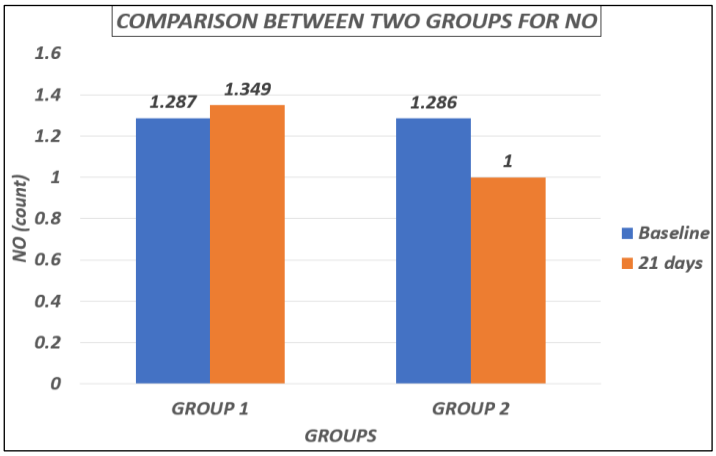
RESULTS

The present interventional parallel design single-blind randomized clinical trial was conducted for an experimental period of 21 days. Clinical and biochemical parameters were comparatively evaluated at baseline and 21 days for GROUP I and GROUP II.

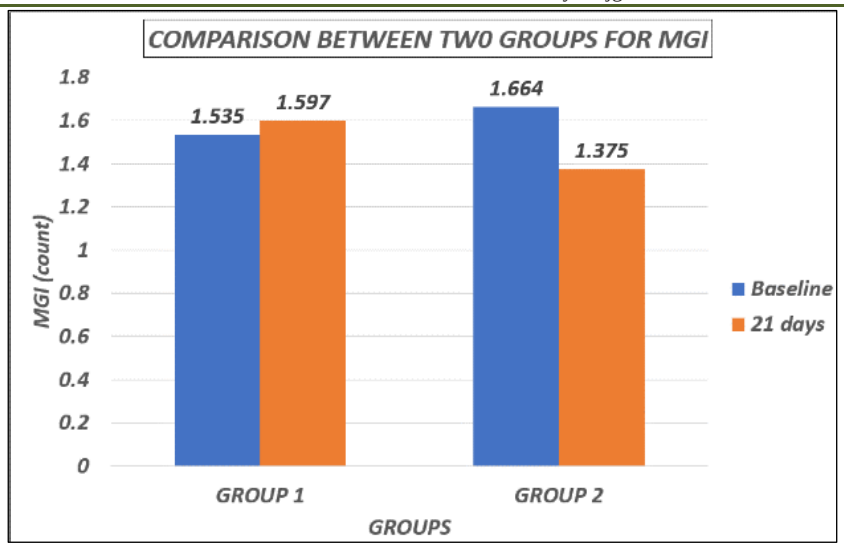
Statistical Analysis

Data was collected and compiled into MS Office excel worksheet & was subjected to statistical analysis using SPSS version 24.0 for Windows (Armonk, NY: IBM corp) Software. In this study, probability of $p \leq 0.05$ was considered as significant. Intergroup comparison of two groups at baseline and 21 days was done using Mann-Whitney Test to evaluate the mean differences according to their distribution.

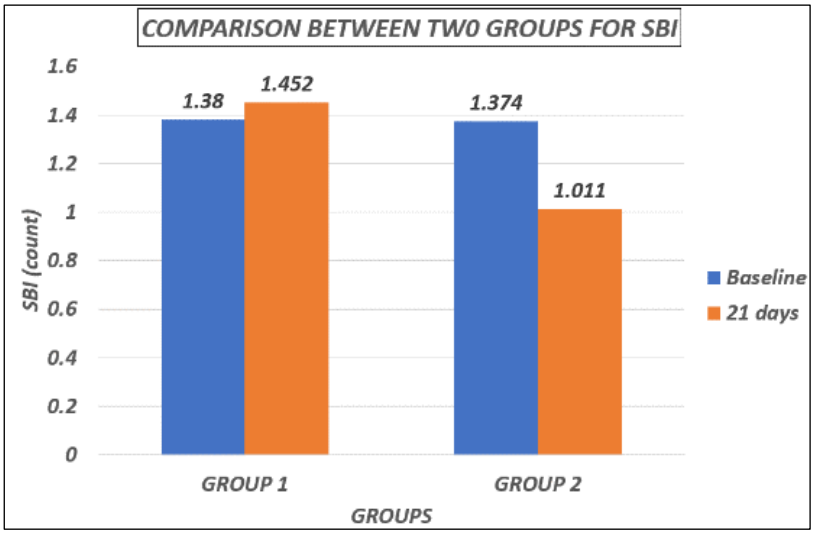
Intergroup comparison demonstrated a significant statistical difference ($p<0.05$) for Group II subjects at 21 weeks than at baseline in terms of Salivary Nitric Oxide levels (Table 1 & 2, Graph 1), Modified Gingival Index (Table 1 & 2, Graph 2), Sulcus Bleeding Index (Table 1 & 2, Graph 3) and Plaque Index (Table 1 & 2, Graph 4) as compared to Group I subjects.



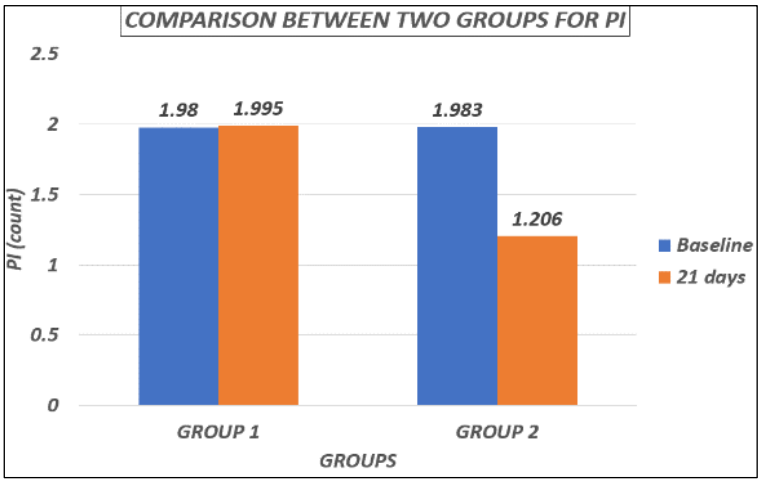
Graph 1: Intergroup comparison for Nitric Oxide



Graph 2: Intergroup Comparison for Modified Gingival Index



Graph 3: Intergroupcomparison for Sulcus Bleeding Index



Graph 4: Intergroup comparison for Plaque Index

Table 1: Descriptive Statistical Analysis

Descriptive Statistics						
GROUPS		**N**	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**
I	Baseline MGI	10	1.39	1.69	1.5350	.10124
	21 days MGI	10	1.40	2.04	1.5970	.18197
	Baseline SBI	10	1.26	1.45	1.3800	.06566
	21 days SBI	10	1.24	1.66	1.4520	.13790
	Baseline PI	10	1.73	2.11	1.9800	.12184
	21 days PI	10	1.23	2.40	1.9950	.35268
	Baseline NO	10	.89	1.99	1.2870	.34808
	21 days NO	10	.65	2.10	1.3490	.45029
II	Baseline MGI	10	1.21	2.23	1.6640	.37134
	21 days MGI	10	1.02	2.12	1.3750	.38991
	Baseline SBI	10	1.45	1.86	1.3740	.13818
	21 days SBI	10	1.44	1.89	1.0110	.16360
	Baseline PI	10	1.74	2.25	1.9830	.15290
	21 days PI	10	1.69	2.02	1.2060	.12057
	Baseline NO	10	1.14	2.19	1.2860	.35775
	21 days NO	10	.89	1.99	1.000	.38353

Table 2 Intergroup comparison by Mann Whitney test

Ranks						
	GROUPS	**N**	**Mean Rank**	**Sum of Ranks**	**X^2**	**P value**
Baseline MGI	I	10	9.90	99.00	44.000	.650
	II	10	11.10	111.00		
	Total	20				
21 days MGI	I	10	11.00	110.00	45.000	.035
	II	10	10.00	100.00		
	Total	20				
Baseline SBI	I	10	115.55	155.50	41.500	.980
	II	10	15.45	154.50		
	Total	20				
21 days SBI	I	10	17.10	171.00	16.000	.010
	II	10	13.90	139.00		
	Total	20				
Baseline PI	I	10	10.60	106.00	49.000	.940
	II	10	10.40	104.00		
	Total	20				
21 days PI	I	10	12.00	120.00	35.000	.026
	II	10	9.00	90.00		
	Total	20				
Baseline NO	I	10	16.80	168.00	13.000	.975
	II	10	14.20	142.00		
	Total	20				
21 days NO	I	10	11.25	112.50	42.500	.041
	II	10	9.75	97.50		
	Total	20				

DISCUSSION

The presented study comparatively evaluated the efficacy of probiotics on salivary levels of nitric oxide in chronic gingivitis patients undergoing orthodontic treatment between the age group of 13-33 years as orthodontic treatment of subjects limited upto this age has been found to show favourable treatment outcomes of orthodontic therapy and moreover; the increased frequency of chronic gingivitis in teenagers and adults in the age group.

In subjects undergoing fixed orthodontic treatment, efficient removal of dental plaque becomes difficult due to the presence of orthodontic appliances such as the brackets and the variability of the different types of bracket designs influences plaque adhesion and makes them difficult to be accessed by mechanical plaque controlling agents. Therefore, the present clinical trial was conducted in orthodontic subjects with chronic gingivitis that involved placement of brackets of variable design such as the Beggs and Preadjusted brackets. Moolya NN *et al*., (2014) [2] established a positive correlation between orthodontic bracket designs and their effect on the amount of plaque formation. They observed a significant impact of Beggs brackets and Preadjusted brackets on the supra-gingival and sub-gingival plaque formation, and clinical periodontal parameters using plaque index (PI) and gingival index (GI).

Roy C Page and Schroeder HE (1976) [13] observed the histopathological events in the pathogenesis of inflammatory periodontal disease and found that the transition of clinically healthy gingiva to chronic gingivitis requires 14-21 days. Also, the healing of diseased periodontal tissue after periodontal therapy that involves resolution of inflammatory changes and subsequent regeneration and reformation of the junctional epithelium and the connective tissue fibres occurs by 3 weeks (21 days) to 4 weeks. Hence, the present study was conducted for a duration of 21 days that was aimed to evaluate the changes in clinical parameters such as Modified Gingival Index (MGI), Sulcus Bleeding Index(SBI), Plaque Index (PI) and biochemical parameter (Salivary Nitric oxide levels) in orthodontic subjects with chronic gingivitis and the effects of phase I periodontal therapy i.e. scaling and root planning (SRP) and probiotic supplement containing Lactobacillus reuteri on gingival inflammation and dental plaque formation.

In the present study, an attempt was made to evaluate the role of salivary nitric oxide as inflammatory biomarker in chronic gingivitis as various studies have reported the significance of nitric oxide as an inflammatory marker. The tissue injury in inflammation involves the production of inducible nitric oxide synthetase (iNOS) that leads to the production of NO. Salivary glands contain nitric oxide synthetase which could explain the source of salivary nitric oxide. Aurer A *et al*., [14] reported that salivary nitrite, a stable metabolite of NO, was decreased in the saliva of the periodontitis patients than in the healthy subjects. Hirose *et al*., (2001) [15] have found that the NO production by macrophages and polymorphonuclear leukocytes via the iNOS pathway resulted in the progression of gingivitis. Thus, the evaluation of nitric oxide in its stable form of nitrite in saliva can help in the assessment of the role of NO in chronic gingivitis. Also, the periodontopathic microorganisms activate iNOS in saliva and other biological fluids to produce NO. Thus, the salivary NO in the form of nitrite levels were evaluated in the present study.

Results of the present study demonstrated a significant positive correlation between Group I and group II at baseline and 21 days. In Group I, comparison of MGI, SBI, PI and salivary levels of NO showed significant reduction at 21 days than at baseline and similarly, for Group II, a significant reduction in terms of MGI, SBI, PI and salivary levels of NO was observed at 21 days as compared to baseline. In a similar study by S. Carossa *et al*., 2001 [16], a significant direct relationship was observed between oral nitric oxide and salivary nitrite during plaque deposition and concluded that oral nitric oxide increases during *de novo* deposition of dental plaque. The reason for increased bleeding on probing, gingival redness, erythema and edema observed GROUP I as compared to GROUP II in context to MGI and SBI can be due to the increased vasodilatory effect of NO which increases vascular permeability and greater inflammatory response.

In this study, adjunctive use of probiotic along with SRP has proved to be more beneficial in the control of gingivitis and plaque formation in GROUP II compared to GROUP I. Plaque index (PI) was found to be greater in GROUP I as compared to GROUP II. This may be due to the interference of increased number of commensal bacteria with the plaque biofilm formation as a result of the adjunctive use of probiotic in GROUP II subjects The regular oral consumption of probiotic capsules containing Lactobacillus reuteri microorganisms by GROUP II subjects could have served as the source of nitrates and nitrites beneficial for the growth of commensal microorganisms along with increase in the number of L.reuteri. The periodontopathic bacteria that are facultative anaerobes can induce the conversion of iNOS present in the saliva into NO and which also also enhance the gastric generation of NO in acidic conditions thus producing a greater inflammatory response and greater tissue destruction by NO free radicals. This conversion can be prevented by the commensal microorganism as they act against the pathogens and prevent the reduction of nitrate and nitrite into nitric oxide. The probiotics thus serve as commensal and prevent the transition of microbial shifts in gingival inflammation. The results were mainly in agreement with the fact that up to 25% of the plasma nitrate is actively taken up by the salivary glands and it is secreted with saliva, and the resulting salivary nitrate concentrations are at least 10 times higher than the concentrations in plasma. These findings are in agreement with a similar study conducted by Margarita Iniesta *et al*., (2012) [17] that evaluated the clinical and microbiological impact of the use of probiotic tablets containing Lactobacillus reuteri and assessed the patterns of L. reuteri colonization in saliva and subgingival biofilm. The results of their study showed a significant reduction in total count of periodontopathogens P. intermedia in saliva and P. gingivalis in subgingival plaque.

The collection of the salivary constituents is a simple, non-invasive procedure that can be performed by any individual and therefore in this study salivary samples were obtained and sent to laboratories for the analysis of NO biomarker. The Griess reaction has potential as an auxilliary diagnostic tool as it is a simple, highly specific and extremely sensitive method for measuring the micromolar concentrations of nitrite present in the samples. Therefore, this method of using Griess reagent can be considered as a reliable technique for salivary nitrite determination.

This is the first study that evaluated the effects of probiotic supplement containing lactobacillus reuteri on salivary levels of nitric oxide, plaque formation, ginigival inflammation and its correlation in orthodontic subjects. The results of our study suggest that the determination of the levels of salivary nitric oxide can even aid in the prediction of the rate of progression of

inflammation in the periodontal tissues. This can be used as therapeutic modality in the prevention of gingival and periodontal disease. As an alternative to antibiotics, the use of probiotics can be been recommended based on their mechanism of action that enhances the commensal flora and impairs the colonization of periodontopathic pathogens.

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CONCLUSION

According to the results of this study, salivary nitric oxide levels prove to be significant marker of gingival inflammation and in addition the adjunctive use of probiotic containing Lactobacillus reuteri can help in the reducing the severity of gingival inflammation and plaque formation in subjects undergoing active orthodontic treatment.

Practical Implications

Probiotic supplement containing Lactobacillus reuteri can prove to be beneficial in reduction of chronic gingivitis in subjects during active orthodontic phase and can be used as treatment protocol therapeutic modality in the prevention of gingival and periodontal disease.

Limitations

Exact mechanism of nitric oxide synthase inhibitors was not evaluated from the present. More clinical trials and advanced research diagnosis is further required for knowing it.

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