Effect of Various Mouthwashes on the Levels of Interleukin-2 and Interferon-γ in Chronic Gingivitis

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The aim of this double blind study was to evaluate the effect of various mouthwashes: Chlorhexidine, Essential oil, Azadirachta indica (Neem) extract, and Povidone iodine on gingival tissue interleukin-2 (IL-2) and interferon-γ (IFN-γ) levels in patients with chronic gingivitis. A total of 80 patients (42 boys, 38 girls; mean age 16.0 ± 1.8 years) were included in this study. Patients were randomly assigned into four groups of 20 each: Group I- Azadirachta indica (Neem) extract, Group II - Essential oil, Group III - Povidone iodine, and Group IV - Chlorhexidine. They were instructed to use these mouthwashes for two weeks. Plaque and gingival indices scores, and IL-2 and IFN-γ levels in the gingival tissues were measured at baseline and after two weeks of mouthwash use. Results showed the reduction of plaque and gingival indices, and IL-2 and IFN-γ level with Chlorhexidine, Essential oil, and Povidone iodine, which were found to be statistically significant. Although Neem reduced the level of plaque and gingival indices, and IL-2 and IFN-γ to a certain level, it was not statistically significant. Therefore, Chlorhexidine, Essential oil, and Povidone iodine mouthwashes can be used as an adjunct to oral prophylaxis in reducing pro-inflammatory cytokines, IL-2 and IFN-γ in patients with chronic gingivitis.

Keywords: *Chronic gingivitis, interleukin-2, interferon-γ, Azadirachta indica, Povidone iodine, Chlorhexidine.*

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INTRODUCTION

Cytokines are potent local mediators of inflammation that are produced at the site of tissue insult or injury. Interleukin-2 (IL-2) is involved in B-cell activation and stimulates macrophages, natural killer cells and T-cell proliferation, which mediate the cellular immune response; thus it is regarded as a pro-inflammatory cytokine.[1] Interferon-γ (IFN-γ) is also a pro-inflammatory cytokine produced by T-helper-1 cells, natural killer cells and macrophages. In addition to the pivotal role of IFN-γ in host defense, its excessive release has been associated with the pathogenesis of chronic inflammatory periodontal disease (CIPD).[2] Both IL-2 and IFN-γ concentrations in inflamed gingival tissues are found to be elevated when compared with healthy sites.[3] Therefore, IL-2 and IFN-γ are considered to be amongst the key cytokines responsible for the initiation and progression of periodontal diseases.[4]

The term periodontal disease refers to all pathologic processes affecting the periodontium. Chronic gingivitis is widespread among children and adolescents. Epidemiologic studies indicate that 80-90% of all children have CIPD by the age of 15 (13-18).[5] This CIPD if left untreated can lead to more destructive forms of the disease later in life. Indeed, recognition of periodontal disease in their earliest stages is the key to their prevention and treatment.

Herbal preparation like *Azadirachta indica* (Neem) extract has been shown to be effective in reducing plaque and gingivitis.[6,7] Essential oil (Listerine) has been found to reduce prostaglandin E2,[8] Povidone iodine for reduction of gingival inflammation,[9] and Chlorhexidine in reducing inflammatory mediators like IL-1β in gingival crevicular fluid (GCF).[10] But there is no study available in the existing literature that these mouthwashes also reduce the levels of IL-2 and IFN-γ in the gingiva in patients having chronic gingivitis.

Therefore, the aim of this study was to evaluate the effect of Neem extract, Chlorhexidine, Povidone iodine, and

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Essential oil mouthwashes on the levels of IL-2 and IFN-γ in patients with chronic gingivitis.

MATERIALS AND METHODS

Study Design

The study was approved by the University Ethical Committee, and informed consent and strict commitment for follow up was obtained after detailed explanation of the treatment to the patients. A total of 80 patients (42 boys and 38 girls) aged between 12-20 years, mean age 16.0 ± 1.8 (Table 1) with chronic gingivitis (score 3),[11] involving at least two sites in different quadrants were selected from the Departments of Pedodontics and Preventive Dentistry, and Periodontics, King George's University of Dental Sciences, Lucknow, India.

Patients with the following conditions were excluded from the study: (1) systemic infections; (2) use of systemic antibiotics or non-steroidal anti-inflammatory drugs in the past three months; (3) complicating systemic conditions, (4) any known allergy to Chlorhexidine, Povidone iodine, Essential oil, and Neem extract, (5) smoking, (6) periodontal treatment undertaken less than 6 months prior to baseline, and (7) patients unable to commit for the follow-up visits.

This was a double blind clinical trial since both the investigator (examiner), and the patients were blinded on treatment. Enrollments of the patients were done only by the investigator to eliminate the selection bias. The coding of the four mouthwashes were done by a co-investigator as: Group I – Neem extract (1% w/v), [National Botanical Research Institute, Lucknow, India]. Group II – Essential oil (Listerine) [Davis (India) Ltd., Hyderabad, India] Group III - Povidone iodine (1% w/v), [Win Medicare, New Delhi, India] and Group IV – Chlorhexidine (0.2%), [ICPA Health Products Ltd., Ankleshwar, India] which were unknown to the investigator as well as the patients. Randomization was achieved by supplying the patients with the numerically coded mouthwashes based on computer generated random numbers.

Plaque scores[12] were recorded. Thereafter, gingival biopsy was taken by the investigator under local anesthesia from buccal interproximal papilla including sulcular epithelium, preferably between second premolar and first molar region. The biopsy sample was washed in distilled water to remove blood clots and debris, put in glass vial, sealed with parafilm, clearly labelled and transported in ice and frozen at –70ºC in a deep freezer until use.

After the biopsy, patients were supplied with triclosan and flouride-free toothpaste and a bottle of the coded mouthwash. They were instructed to rinse thoroughly for one minute, half an hour after tooth brushing with 10 ml of the given mouthwash (without any dilution), twice a day (morning and night) for two weeks. They were asked to avoid taking anything orally for about 30 minutes after the rinse. Patients were also instructed not to use any systemic medications for the study period of two weeks. All the patients were instructed to report on the 3rd day to reinforce the given instructions. On the 15th day of recall visit, plaque and gingival scores were again recorded, and second gingival biopsy was obtained from another quadrant already selected at the baseline visit. Two different sites were chosen for biopsy in order to avoid inadequately healed first biopsied area thereby preventing false estimation of cytokines. This sample was also prepared and stored as the baseline sample until use.

In the laboratory each sample was thawed for 30 minutes and weighed. It was then finely minced with scalpel blade and diluted in cold phosphate buffered saline (PBS) in the ratio of 100 mg in 1 ml of PBS solution. The tube containing the sample was placed in a homogenizer (by placing in an ice beaker for 3 minutes). The homogenized sample was put in an eppendorf tube and centrifuged at 370xg for 5 minutes, and the supernatant was divided equally into two parts for determining cytokine (IL-2 and IFN-γ) levels, which was carried out by ELISA kits (Biotrak, Amersham International plc, Buckingham, England) [R and D system] strictly following the manufacturer's instructions. These cytokines levels were detected by quantitative "sandwich" enzyme immunoassay technique. Recombinant human IL-2 and IFN-γ were used as standards. The optical densities of the samples were determined using an ELISA reader set at 450 nm.

The concentrations of IL-2 and IFN-γ in the unknown samples were then determined by comparing the optical density of the samples to the standard curve. The cytokine content of each sample is expressed in pg/ml of tissue extract.

Statistical Analysis

Data were entered into MS-excel. Statistical Package for Social Sciences (12.0) was used to analyze the data. Qualitative variables were compared with Chi-square test. Student t-test and F-test were used to compare the mean values for continuous variables, and Wilcoxon Signed Ranks test was used for skewed data. All 'p' values were two tailed and values less than 0.05 were considered as significant.

Table 1. Age-Sex distribution of Patients

Mouthwash(Group)	No. of Patients	Sex		Age	
		Male	Female	Range	Mean ± SD
Neem extract (I)	20	9	11	15-20	16.4 ± 1.5
Essential oil (II)	20	11	9	14-20	16.6 ± 1.8
Povidone iodine (III)	20	12	8	12-19	15.4 ± 1.9
Chlorhexidine (IV)	20	10	10	13-18	15.5 ± 1.6
Total	80	42	38	12-20	16.0 ± 1.8
Significance		χ^2 =1.00, p=0.80		F = 2.68, p=0.052	

RESULTS

All the 80 patients completed two weeks of study period. Patients of group I (Neem extract) complained about bitter taste. Few patients of group IV (Chlorhexidine) complained

Table 2. Plaque and Gingival Indices (mean ± SD) of the biopsy areas

Scores		Neem extract (n=20)	Essential oil (n=20)	Povidone iodine (n=20)	Chlorhexidine (n=20)
Plaque Index	Before	2.32±0.91	2.25±0.52	1.53±0.37	1.94±0.21
	After	2.01±0.78	1.72±0.24	1.29±0.21	1.12±0.36
	Significance	t=1.16, p>0.05	t=4.14, p<0.001	t=2.52, p<0.02	t=8.8, p<0.001
Gingival Index	Before	2.84±0.81	2.88±0.75	2.85±0.71	2.82±0.83
	After	2.42±0.63	1.92±0.23	2.25±0.56	1.51±0.42
	Significance	t=1.83, p>0.05	t=5.47, p<0.001	t=2.97, p<0.005	t=6.25, p<0.001

Table 3. Reduction in IL-2 and IFN-γ levels (pg/ml) after two weeks of mouthwash use

Mouthwash	IL-2 (Median)			IFN-γ (Median)		
	Before	After	p value	Before	After	p value
Neem extract (n=20)	560.00	540.00	0.080	2.00	1.85	0.120
Essential oil (n=20)	545.00	503.00	0.001	3.27	2.45	0.001
Povidone iodine (n=20)	557.50	323.50	0.013	3.05	2.50	0.004
Chlorhexidine (n=20)	608.00	473.00	0.000	3.05	2.13	0.000

of loss of taste sensation during the study period. After two weeks of mouthwash use, groups II, III, and IV showed significant decrease for plaque ($p<0.001$, $p<0.020$, and $p<0.001$), and gingival indices ($p<0.001$, $p<0.005$, and $p<0.001$), respectively (Table 2). The reduction of IL-2 and IFN-γ levels were highly significant ($p<0.000$) with group IV (Table 3). Group II and III also showed significant reduction of both the cytokines, IL-2 ($p<0.001$ and $p<0.013$), and IFN-γ ($p<0.001$ and $p<0.004$), respectively. Though group I also reduced plaque and gingival indices scores, and IL-2 and IFN-γ to a certain level, however, they were not statistically significant.

DISCUSSION

Gingival inflammation is caused by plaque forming bacteria. In this inflammatory process endotoxins are released that stimulate variety of cells which induce synthesis of pro-inflammatory cytokines.[4] These cytokines play an important role in the initiation and progression of inflammatory periodontal diseases.[2] Cytokines that are present in GCF and inflamed gingiva have been investigated as potential diagnostic markers of periodontal inflammation.[13]

Periodontal disease is an extremely slow process, the early stages of which are common during puberty. Unless these early stages are diagnosed and treated, degenerative periodontal disease is inevitable in later years wherein methods of treatment are seldom effective. In childhood, therefore, periodontal disease has already begun; it is most important to recognize and to treat it. Chemical methods of plaque control act as a very good adjunct to mechanical means in children and adolescents wherein plaque control is not maintained effectively.

Chlorhexidine is a chlorophenyl bi-guanide with broad antimicrobial activity. Loe and Schiott[14] reported highly significant inhibition of plaque formation and prevention of gingivitis by the use of an aqueous solution of 0.2% Chlorhexidine as a mouth-rinse twice daily for one minute. Essential oil mouth-rinse is the oldest and the most widely used antiseptic agent with anti-plaque and mild anti-inflammatory activity.[8] Povidone-iodine is an effective antibacterial agent when used as a mouthwash, although long term improvement in gingival health has not been substantiated.[15]

Neem has been used since ages in different forms in the treatment of oral diseases because of its medicinal value. It is one of the most researched tropical trees with almost all its parts being put for a variety of uses.[6,7,16] Though different studies have shown antimicrobial, anti-plaque, and anti-inflammatory properties of these aforementioned mouthwashes, their effect on gingival tissue cytokines has not been reported yet. This prompted us to undertake this study on two of the cytokines responsible for initiation and progression of periodontal diseases.[4]

The clinical parameters like bleeding on probing, color and texture changes are most commonly used to evaluate gingival inflammation or the pathologic effects of inflammation on periodontal tissues.[17] The interpretation of these objective (bleeding on probing), and subjective (color and texture) changes vary from clinician to clinician. Therefore, diagnostic assessment of biologic markers like cytokines are more reliably used to determine the exact health status of the periodontium.[13] It is documented that cytokines like IL-2 and IFN-γ are very high in inflamed gingiva.[18,19] It has also been accepted that change in the severity of disease is accompanied by a concomitant change in the levels of the pro-inflammatory cytokines.[19]

The previous studies[10,20] have generally compared the levels of cytokines in tissues or GCF of healthy subjects with those having periodontitis. Ebersole *et al*.,[19] compared inflammatory mediators pattern regulated by host responses at individual sites of periodontal health, gingivitis and periodontitis where they demonstrated high levels of cytokines (IL-1, IL-6 and IL-2) in gingivitis, whereas no particular unique cytokines profile was noted at the time of development of periodontitis. These findings suggest that alterations in levels of IL-2 and IFN-γ may initiate the stages of gingivitis and relate more directly to the inflammatory response which if not treated may progress to periodontitis. Seymour and Gemmell[18] also in their review article concluded that within the tissue, gingivitis is associated with T-helper-1 response (IL-2 and IFN-γ) while periodontitis is associated with T-helper-2 response (IL-4, IL-5, IL-6 and IL-10). This point favors the selection of IL-2 and IFN-γ for assessing chronic gingivitis status in this study.

Importance was given to continue with the same oral

hygiene measures as they were doing it before so as to avoid any change in the cytokines levels due to the change in the oral hygiene habits rather than by the use of mouthwashes. Triclosan and fluoride-free toothpaste was chosen to avoid its antiplaque effect.

After two weeks of mouthwash use, significant clinical improvement in plaque and gingival indices scores were shown by Essential oil, Povidone iodine, and Chlorhexidine. It was also found that reduction in plaque and gingival scores were accompanied by significant decrease in the levels of pro-inflammatory cytokines (IL-2 and IFN-γ) by the same mouthwashes. Chlorhexidine showed highly significant reduction of both the cytokines among all the other mouthwashes used. However, contrary to the study of Pai et al.,[7] the reduction of plaque and gingivitis with Neem extract seen in our study was statistically insignificant.

From a biochemical perspective, proteins are physically altered or denatured to some extent by repeated freezing and thawing. All experimental samples analyzed in this study were subjected to standardized collection, storage and processing procedures as mentioned in Materials and Methods section. If some experimental protein was lost during the sample preparation phase as a result of freezing and thawing, it was a common factor for all samples.

Another experimental concern relates to whether complete elution of IL-2 and IFN-γ could have been accomplished with the procedures used in this study. The ability of mincing and crushing procedures to completely liberate IL-2 and IFN-γ from the tissue sample is unknown. Therefore, it is probable that the prepared supernatant fluid contains less IL-2 and IFN-γ than the intact gingival tissue sample. However, care was taken to uniformly mince and crush the experimental tissue in PBS by the same investigator. With these precautions, the amount of IL-2 and IFN-γ release from the tissues by the standard preparation technique was believed to be uniform for all experimental samples.

CONCLUSIONS

The data in the present study suggested that except Neem extract, the other mouthwashes used in the study namely: Chlorhexidine, Essential oil, and Povidone iodine reduced plaque and gingival inflammation along with IL-2 and IFN-γ levels significantly from gingival tissues in patients with chronic gingivitis. Among the mouthwashes used, Chlorhexidine showed highly significant reduction of these cytokines demonstrating a very important role in the treatment of chronic gingivitis. However, further studies are required to clarify the effect of these mouthwashes on the levels of IL-2 and IFN-γ along with the elucidation of its biological mechanisms.

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