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Effect of 0.12% Chlorhexidine Gel and Tooth Brushing on *Porphyromonas Gingivalis* Levels in Subgingival Plaque and Matrix Metalloproteinase-8 (MMP-8) Levels in Gingival Crevicular Fluid of Children and Adolescents with Down Syndrome

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Aim: This study aims to determine if chlorhexidine gel (0.12%) and tooth brushing had any effect on the levels of MMP-8 in GCF and the levels of Porphyromonas gingivalis in subgingival plaque of DS children and healthy children.

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Aim: This study aims to determine if chlorhexidine gel (0.12%) and tooth brushing had any effect on the levels of MMP-8 in GCF and the levels of Porphyromonas gingivalis in subgingival plaque of DS children and healthy children.

Methodology: Twenty Down syndrome and twenty healthy children formed Group I and Group II, respectively. Following the collection of baselines GCF samples and plaque samples, oral prophylaxis was carried out by the same examiner in both groups. In Group I, chlorhexidine (0.12%) gel was applied over the gingiva once in two weeks for 3 months. Parents were given oral hygiene counseling on the method of tooth brushing for their children. A second sample of GCF and plaque was obtained from Group I at the end of 3 months. Oral hygiene reinforcement was given for the children once in two weeks for the next three months and the third sample of GCF and plaque was obtained from Group I at the end of the 6th month.

Results: A significant difference was observed in mean MMP-8 levels and mean *Porphyromonas gingivalis* levels between Down syndrome and healthy children ($p<0.001$).

Conclusion: The levels of MMP-8 and Pg. in Down syndrome children showed improvement following the use of 0.12% chlorhexidine gel.

Keywords: down syndrome, prevention, chlorhexidine, periodontal health, gingival health.

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I. Introduction

Down syndrome is a genetic disorder caused by the presence of all or part of an extra 21st chromosome and is also known as trisomy 21. Intellectual disability, cardiac anomalies and an altered immune system in individuals with Down syndrome can have a profound effect on their oral health.[1] Children with Down syndrome (DS) experience a high incidence of rapid, destructive periodontal disease, which may be related to local factors such as tooth morphology, bruxism, malocclusion, and poor oral hygiene as well as systemic factors such as altered immune/inflammatory responses. Early colonization of periodontal pathogens is another important contributing factor to their increased susceptibility to periodontitis. 2-4 Among the pathogens, *Porphyromonas gingivalis* is the predominant pathogen seen in subgingival dental plaque of DS adolescents.[4]

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, which have been considered as mediators of extracellular matrix degradation and remodeling during periodontal diseases. The host response thought to be caused by interactions between dental plaque, calculus, and oral bacteria, leads to inflammatory cascades involving increased activities of proteolytic enzymes in gingival tissue, gingival crevicular fluid, and saliva. The expression of MMPs and their levels in the gingival crevicular fluid are good indicators for a clinical diagnosis of periodontal disease.[3] Matrix metalloproteinase-8 is a poly morpho nuclear leukocyte (PMN)-type collagenase involved in periodontal tissue degradation in periodontal disease. It is stored in specific granules within PMNs and is released when PMNs are triggered. MMP-8 is the major collagenase involved in periodontitis.

Thus, the levels of *Porphyromonas gingivalis* and MMP-8 are good clinical indicators of periodontal disease in these adolescents. Supervised preventive

programs have been very effective in reducing plaque and gingival inflammation in people with Down Syndrome.[5] The use of antimicrobial agents can be a useful aid in plaque control for these individuals.[6]

Most of the studies on the oral health of DS children and adolescents have reported on their salivary parameters, dental caries and oral hygiene.6-8 Therefore, the objective of this clinical study was to comparatively evaluate MMP-8 levels in gingival crevicular fluid and *Porphyromonas gingivalis* in the dental plaque of subjects with Down syndrome and healthy controls. The aim was also to assess whether tooth brushing, with or without the use of chlorhexidine, influences the levels of MMP-8, *Porphyromonas gingivalis*, dental plaque and gingival health of subjects with DS.

II. Methodology

This randomized controlled trial included twenty children and adolescents, aged 9-16-year-old with Down Syndrome selected from Divya Downs Developmental Trust, Bangalore. Before the study, ethical approval and clearance were obtained from the Institutional Ethics Review Board of our institution. A written permission had been obtained from the concerned authorities of the school. The nature of the study was explained to the authorities, and prior informed written consent had been taken from the parents/caretakers of all the subjects. A proforma had been used to gather demographic data, medical and drug history. The exclusion criteria were: (1) those on long term medication, (2) those who were very uncooperative and were unable to cooperate, (3) severe intellectual disability present along with Down syndrome, (4) association with any other medically compromised conditions and (5) those who had undergone oral prophylaxis in the preceding six months.

Twenty healthy controls who were age and gender-matched had been selected from the Department of Pedodontics and Preventive Dentistry at our institution, to obtain estimates of MMP-8 and *Porphyromonas gingivalis* for comparison. Therefore, there were two groups: Group I: Twenty subjects with Down syndrome and Group II: Twenty healthy subjects.

Oral examination was done by a single trained and calibrated examiner under artificial light using a sterile dental mirror and WHO CPITN probe. In Group I, the plaque index (PI) had been recorded using *Silness and Loe* index9 and gingival health had been assessed using *Loe and Silness* gingival index (GI).10

a) Collection of baselines gingival crevicular fluid (GCF)

Gingival crevicular fluid (GCF) samples (2µl) were collected using sterile microcapillary pipettes (0.01mm). Pooled GCF samples had been collected from the mesial surfaces of first permanent molars by placing the tip of the micropipette at the entrance of the gingival sulcus for 30 seconds. Matrix metalloproteinase-8 (MMP-8) levels were analyzed from the samples that were wrapped in aluminum foil, stored at -80°C.[11]

b) Assessment of matrix metalloproteinase-8

The levels of matrix metalloproteinase-8 (MMP-8) were estimated by subjecting the collected GCF samples to enzyme-linked immunosorbent assay (ELISA).11ELISA was performed based on the protocol in the Fine test Human Matrix Metalloproteinase-8 kit (Wuhan Fine Biological Technology Co., Ltd.).The plate was washed twice before adding standard, sample, and control(zero) wells. 0.1ml of solution was then aliquoted into 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.3125ng/ml, 0.156ng/ml, standard solutions into the standard wells. 0.1ml of sample/ standard dilution buffer was added into the control well. 0.1ml of the test samples were added into test sample wells. The plate was sealed and incubated at 37°C for 90 minutes. The excess fluid was removed using absorbent filter papers. 0.1ml of biotin-detection antibody working solution was added into the above well; the plate was sealed and incubated at 37°C for 60 minutes. The plate was then washed thrice with wash buffer. 0.1ml of HRP-Streptavidin Conjugate (SABC) working solution was added into each well, and the plate was sealed and incubated at 37°C for 30 minutes. The seal was then removed, and the plate content was discarded, the plate was then washed 5 times with wash buffer. 90µl of tetra methyl benzidine (TMB) substrate was added into each well. The plate was sealed and incubated at 37°C in the dark for 15-30 minutes. Stop solution (50µl) was added into each well and mixed thoroughly. The color changed to yellow immediately. The optical density (OD) absorbance was read at 450nm on a microplate reader immediately (within 30 minutes) after adding the stop solution. The calculation was done as: (The relative OD 450) = (the OD 450 of each well) – (the OD 450 of Zero well). The MMP-8 concentration of the samples was interpolated from the standard curve.

c) Collection of baseline subgingival plaque

Subgingival plaque samples were collected from the mesial and buccal sites of first permanent molars using sterile curettes. The plaque samples had been then transferred into sterile Eppendorf tubes containing buffer solution, stored at -80° C for further analysis of *Porphyromonas gingivalis*.[12]

d) Assessment of Porphyromonas gingivalis

The presence of *Porphyromonas gingivalis* had been microbiologically evaluated by suspending the plaque sample in 1ml of saline. Aseptically 0.1ml of the suspension was transferred to Tryptic soy broth (TSB culture medium) and incubated under anaerobic conditions overnight for 24 hours at 37°C. The growth of the bacteria was measured spectrophotometrically by

reading its optical density (OD) at 600-nanometer wavelength and expressed as colony forming units (CFU) per ml.[13]

Following the collection of baseline plaque and GCF samples, oral prophylaxis was carried out in both groups.

e) Preparation of Chlorhexidine (0.12%) gel

A gel consisting of hydroxy propyl methylcellulose (HPMC), glycerin and water was prepared. Water was added to HPMC, followed by vigorous mixing until the HPMC became miscible with water. Glycerin was then added to this mixture and mixed well to form a gel. Further, a commercially available chlorhexidine (CHX) gel (2%) (Unilab Chemicals and Pharmaceuticals Pvt. Ltd., India) was added to this gel and stirred using a magnetic stirrer to obtain 60 g of 0.12% CHX gel.

f) Periodic application of the Chlorhexidine (0.12%) gel for the first three months

Following oral prophylaxis, this indigenously prepared CHX gel (0.12%) was applied only in Down syndrome subjects, by gently massaging 0.3-0.5g of the gel over the buccal and palatal/lingual surfaces of gingiva using sterile cotton swabs. The time of application was between 10 am to 11 am. They were instructed not to drink, eat, or rinse their mouth for 30 minutes following the gel application. Regular application of CHX gel was carried out by the same examiner once in 15 days over 3 months. A total of 6 applications were done. During this period, parents and the children were given oral health education inside the school premises and were taught the proper methods of tooth brushing.

g) Collection of the second sample of GCF and subgingival plaque

At the end of 3 months, the second sample of GCF and the subgingival plaque was collected only from the Down syndrome subjects (Group I) in the same manner as described earlier.

h) Oral hygiene reinforcement for the next three months

Oral hygiene reinforcement comprising of oral health education on proper tooth brushing techniques followed by a demonstration of the same was carried out for the parents as well as the children at their school premises. It was carried out by the same examiner once in 15 days over the next 3 months.

i) Collection of the third sample of GCF and subgingival plaque

At the end of the sixth month, after successful completion of periodic oral hygiene reinforcement, a third sample of GCF and the subgingival plaque was collected from all the subjects in the same manner.

j) Tests used for statistical analysis

Data obtained was tabulated and subjected to statistical analysis using Independent student t-Test for the comparison of mean MMP-8 and *Porphyromonas gingivalis* levels between control and Down syndrome group, Friedman's Test for the comparison of mean MMP-8 levels between different time intervals in Down syndrome group, Wilcoxon Signed Ranked Post hoc Test had been used for the pairwise comparison of mean difference in MMP-8 levels between different time intervals, repeated measures of ANOVA Test had been used for the comparison of mean *Porphyromonas gingivalis* optical density levels between different time intervals in Down syndrome group and Bonferroni's Post hoc Test had been used for the pairwise comparison of mean difference in *Porphyromonas gingivalis* optical density levels between different time intervals. Pearson's correlation had been used to study the association between MMP-8 and *Porphyromonas gingivalis*.

III. Results

The mean levels of baseline MMP-8 in Group I was 1.253±0.268 ng/ml, which was significantly higher than that of 0.028±0.022 ng/ml in Group II ($p<0.001$). The mean levels of baseline *Porphyromonas gingivalis* in Group I was 0.317±0.035 CFU/ml, which was significantly higher than that of 0.048±0.04 CFU/ml seen in Group II ($p<0.001$) (Table 1). There was a significant difference observed in the mean levels of MMP-8 and the mean levels of *Porphyromonas gingivalis* at different time intervals($p<0.001$) (Tables 2 and 3). Pair-wise comparison of MMP-8 levels in GCF at different time intervals in Group I showed a significant difference between baseline and three months and between baseline and six months ($p<0.001$) (Table 4). Pair-wise comparison of *Porphyromonas gingivalis* in dental plaque at different time intervals in Group I showed a significant difference between baseline and three months (Table 5).

A significant difference was observed in the mean plaque index (PI) and mean gingival index (GI) scores at different time intervals in Group I (Table 6). Pair-wise comparison of mean plaque index (PI) scores and mean gingival index scores (GI) at different time intervals in Group I showed a significant difference between baseline and three months and between baseline and 6 months (Tables 7 and 8). Pearson's correlation showed a significant association between MMP-8 and *Porphyromonas gingivalis* at three months (Table 9).

IV. Discussion

Individuals with Down syndrome are more likely to develop the aggressive periodontal disease at an earlier age than the general population.4,14,15 Subjects with severe intellectual disability and very uncooperative

children were excluded from the study to facilitate the collection of samples. Sampling was done only after establishing a friendly relationship between the examiner and subjects. Alterations in levels of MMP-8 and *Porphyromonas gingivalis* were avoided by including only those DS individuals who had not undergone oral prophylaxis in the preceding six months.

Dental plaque was recorded using Silness and Loe plaque index because it ignores the coronal extent of plaque and assesses only the thickness of plaque at the gingival area of the tooth. It has good validity and reliability for both mechanical anti-plaque procedures and chemical agents. Loe and Silness gingival index were used because it is simple to use, reliable and can determine the severity of gingivitis. [9,10]

A sensitive and specific marker for periodontal tissue destruction is gingival crevicular fluid. It can be obtained in a non-invasive manner with minimal discomfort to the patient and consists of both locally synthesized and systemically derived molecules.[16] In this study, pooled GCF samples were collected from the mesial surfaces of first permanent molars, as these teeth are the first permanent teeth to erupt and are susceptible to periodontitis.

Enzyme-linked immunosorbent assay (ELISA) was used in this study for the quantification of MMP-8. This assay measures total collagenase i.e. pro- and active enzyme as well as collagenase in complex with Tissue inhibitors of metalloproteinase (TIMP). A fundamental problem with bioassays is that they lack specificity, i.e. they are unable to distinguish between two enzymes that degrade the same substrate, thus leading to inaccurate measurements with elevated enzyme levels.[17] Therefore, a commercially available ELISA kit specific for MMP-8 and with good sensitivity was used.[18]

Spectrophotometric analysis was employed to assess the growth of *Porphyromonas gingivalis.* It is estimated by optical density, which is based on the fact that an increase in the number of bacteria, results in less light transmitted. It is a simple, rapid, low cost and non-destructive method.[19]

In individuals with DS, there is a need for an oral hygiene regime that is simple, easy, economical and acceptable to both patient and caregiver.[20] Cumbersome tooth brushing techniques, and flossing, may be difficult to practice, due to reduced manual dexterity.21 Anti-plaque chemical agents such as chlorhexidine gluconate along with tooth brushing has proved to be useful in reducing plaque and gingivitis.[21] However, how it is delivered may be critical to a successful outcome.[22] Mouth rinses may not be suitable for use in DS, due to their inability to rinse the mouth and low gag reflex. The Application of CHX gel in trays has not been well accepted in children with intellectual disabilities.[21] Higher concentrations of CHX (0.2% or 1%) have been reported to cause mucositis, superficial mucosal erosions and burning sensation.18 In this study, a structured plaque control regime was implemented in subjects with DS. Tooth brushing which is a simple yet effective method for reducing plaque and gingivitis was followed. Good compliance was achieved by using a convenient and simple technique of massaging 0.12% CHX gel over the gingiva. CHX, a cation, interacts and forms salts of low solubility with anions, such as sodium lauryl sulphate (SLS) and sodium monofluorophosphate (MFP) present in dentifrices. To optimize the anti-plaque effect of CHX, an interval of 2 hours was given between tooth brushing and application of CHX gel.23 Long term use of CHX has side effects like extrinsic staining,[18] offensive taste, and altered taste sensation. Hence, the gel application was carried out fortnightly, and only for an initial period of three months.

Several studies have shown higher plaque accumulation and greater severity of gingivitis in DS children compared to healthy children.[6,7,24] In the present study, there was a significant decrease of 35.4% in plaque scores from 1.47 to 0.95, at the end of three months. On discontinuation of CHX gel application, plaque score increased by 28.4% to 1.22. Similarly, moderate gingivitis (GI score=1.48) that was seen at the beginning showed a significant reduction in inflammation resulting in mild gingivitis (0.99) after three months. Subsequently, due to the increase in plaque, there was a reversal to signs of moderate gingivitis (1.24) at six months.

The baseline MMP-8 level in GCF of DS (1.253 ng/ml) was significantly higher than that of healthy controls (0.028 ng/ml), which is in accordance with earlier reports.[11,12] This is probably due to the increased inflammatory response seen in DS and due to the presence of gingivitis. There was a significant eight-fold reduction in the MMP-8 level in subjects with DS after three months of following both mechanical and chemical plaque control. CHX inhibits the activities of MMP-8 thereby, indicating its anti-proteolytic properties.[25] On discontinuation of CHX gel application, MMP-8 level was seen to increase by 16.65% to 0.413 ng/ml.

Our study found the levels of *Porphyromonas gingivalis* to be significantly higher in DS. This microorganism thrives in individuals with poor oral hygiene and high plaque accumulation. *Porphyromonas gingivalis* stimulates periodontal cells to produce inflammatory mediators such as prostaglandin E_2 (PGE_2), matrix metalloproteinases (MMPs), and proinflammatory cytokines including interleukin (IL)-1, IL-6 and IL-8.[26] An association between *Porphyromonas gingivalis* and MMP-8 was also seen in our study.

In the present study, tooth brushing along with CHX gel application in subjects with DS decreased *Porphyromonas gingivalis* by 38.9% (from 0.316 CFU/ml to 0.193 CFU/ml) at the end of three months. This significant reduction was not long-lasting, because the

absence of CHX application during the next three months, resulted in a 47.7% increase in *Porphyromonas gingivalis*.

Gingival massaging of CHX gel can mechanically disrupt the biofilm on teeth, dispersing the agents throughout the gingiva, stimulating blood circulation to the gingival tissues and thereby strengthening its immune response. The most important unique property of CHX is its substantivity or oral retentiveness.[27] CHX also has the ability to neutralize *Porphyromonas gingivalis*. The di-cationic positively charged CHX is attracted to the negatively charged phosphate containing compounds in the bacterial cell wall. This alters the integrity of the bacterial cell membrane and makes CHX get attracted to the inner cell membrane and binds to phospholipids causing leakage of low molecular weight compounds like potassium ions. The cytoplasm of the cells get coagulated and chemically precipitated due to the formation of phosphate complexes which include adenosine triphosphate and nucleic acids leading to bacterial death.[28]

Individuals with DS need more assistance from caretakers with their daily oral health care. In our study, reinforcement of tooth brushing through instructions, monitoring, and continuous motivation was carried out in the presence of parents/ caregivers and schoolteachers. The visits were interactive, and parents discussed their child's oral health.

The results of this study indicated that professional treatment along with regular tooth brushing and CHX gel (0.12%) application for a short duration brought about an improvement in gingival health. However, in order to obtain long-lasting effects, periodic application of CHX gel in low concentration may be necessary in addition to mechanical plaque control.

V. Conclusion

The levels of MMP-8 in GCF and levels of *Porphyromonas gingivalis* in subgingival plaque of Down syndrome children were significantly higher than that of healthy children. The gingival health of Down syndrome children showed improvement following tooth brushing, use of 0.12% chlorhexidine gel and oral hygiene reinforcement. It indicates the need for continuous mechanical and chemical plaque control measures along with regular monitoring in Down syndrome children.

Legends of Tables

Table 1: Comparison of mean levels of MMP-8 and *Porphyromonas gingivalis* between the groups

Group	MMP-8 levels Mean ± SD (ng/ml)	*Porphyromonas gingivalis* levels Mean ± SD (CFU/ml)
Down syndrome (Group I)	1.253 ± 0.268	0.317 ± 0.035
Control (Group II)	0.028 ± 0.022	0.048 ± 0.04-
t	20.375	22.474
p value	<0.001*	<0.001*

*p<0.001 is significant

Table 2: Comparison of mean MMP-8 levels at different time intervals in Group I

Time	MMP-8 levels Mean ± SD (ng/ml)	H	p value
Baseline	1.253 ± 0.268	34.300	p<0.001*
At 3 months	0.155 ± 0.147		
At 6 months	0.413 ± 0.319		

*p<0.001 is significant

Table 3: Comparison of mean *Porphyromonas gingivalis* levels at different time intervals in Group I

Time	*Porphyromonas gingivalis* levels Mean ± SD (CFU/ml)	H	p-value
Baseline	0.3167±0.035	61.819	p<0.001*
At 3 months	0.1936±0.051		
At 6 months	0.2858±0.044		

*p<0.001 is significant

Table 4: Pair-wise comparison of MMP-8 levels in gingival crevicular fluid at different time intervals in Group I

Time	Time	MMP-8 levels Mean difference (CFU/ ml)	95% confidence interval		p-value
			Lower	Upper	
Baseline	At 3 months	1.0970	0.914	1.280	<0.001*
	At 6 months	0.8390	0.560	1.118	<0.001*
At 3 months	At 6 months	-0.2580	-0.435	-0.081	0.001

*p<0.001 is significant

Table 5: Pair-wise comparison of *Porphyromonas gingivalis* in dental plaque levels at different time intervals in Group

Time	Time	*Porphyromonas gingivalis* levels Mean difference (CFU/ ml)	95% confidence interval		p-value
			Lower	Upper	
Baseline	At 3 months	0.1230	0.0900	0.1560	<0.001*
	At 6 months	0.0310	0.0060	0.0560	0.01
At 3 months	At 6 months	-0.0920	-0.1240	-0.0610	<0.001*

*p<0.001 is significant

Table 6: Comparison of mean plaque index (PI) and gingival index (GI) scores at different time intervals in Group I

Index	Time			H	p-value
	Baseline	At 3 months	At 6 months		
Plaque Index Score (Mean± SD)	1.47 ± 0.36	0.95±0.28	1.22±0.29	38.100	<0.001*
Gingival Index Score (Mean± SD)	1.48 ± 0.35	0.99 ± 0.38	1.24 ± 0.39	39.077	<0.001*

*p<0.001 is significant

Table 7: Pair-wise comparison of mean plaque index (PI) scores at different time intervals in Group I

Time	Time	Plaque index score Mean difference	95% CI for Diff.		p-value
			Lower	Upper	
Baseline	At 3 months	0.52	0.42	0.62	<0.001*
	At 6 months	0.25	0.16	0.34	<0.001*
At 3 months	At 6 months	-0.28	-0.34	-0.21	0.001

*p<0.001 is significant

Table 8: Pair-wise comparison of mean gingival index (GI) scores at different time intervals in Group I

Time	Time	Gingival index score Mean difference	95% CI for Diff.		p-value
			Lower	Upper	
Baseline	At 3 months	0.50	0.42	0.57	<0.001*
	At 6 months	0.25	0.18	0.31	<0.001*
At 3 months	At 6 months	-0.25	-0.29	-0.21	0.001

*p<0.001 is significant

Table 9: Association between MMP-8 levels and *Porphyromonas gingivalis*

Time	r score	p value
At Baseline (DS)	0.115	0.315
At 3 months	1.000	0.000*
At 6 months	-0.047	0.422

*p<0.001 is significant

Why this paper is important to Pediatric dentists?

- This study indicates the need for continuous mechanical and chemical plaque control measures in Down syndrome children.
- Pediatric dentists can opt for a 0.12% Chlorhexidine gel to be implemented in their practice for these children.
- Pediatric dentists can regularly educate the parents and children and conduct oral hygiene reinforcements in these children.

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