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Effect of different surface treatment protocols on adherence of streptococci mutans to enamel: An Invitro study

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Abstract

Background: Streptococci mutans has been documented to be the causative microorganism for initiation of dental caries and is an integral constituent of the cariogenic biofilm. Challenging the oral microbiome by various surface treatment modality and preventing the adherence of the causative cariogenic organism is an area which needs to be researched.

Material & Methods: 104 freshly extracted molars were collected. After disinfection, the occlusal surface was decoronated with a diamond disc. The occlusal surface samples were randomly divided into eight groups which are as follows: Group A: Control - No surface treatment; Group B: Laser irradiation of 3.5 watts of 810nm of Aluminium Gallium Arsenide laser for 30 sec; Group C: 1.23 % APF gel; Group D: Re-mineralizing paste (CPP ACP F); Group E: IgY; Group F: Laser irradiation 3.5 watts for 30 sec followed by fluoride treatment of 1.23 APF gel; Group G: Laser irradiation 3.5 watts for 30 sec followed by application of remineralizing paste CPP ACP F; Group H: Laser irradiation of 3.5 watts followed by treatment with IgY. ATCC strain of Streptococcus mutans (25175) was procured (HI Media Laboratories Pvt. Limited) and sub-cultured as per standard guidelines. The purity of cultures was confirmed by staining and biochemical reactions. The organism was then sub-cultured in Brain Heart Infusion broth (BHI) and incubated at 37°C to adjust the suspension to 0.5 Mac

Farland standard, which corresponds to 108 colony-forming units per millilitre (CFU/ml). The teeth samples were immersed in the broth in separate sterile containers and agitated at 80 rpm for 90 minutes to promote adhesion of the organism on the tooth surface. Following this, the broth was aspirated and teeth samples were washed twice with sterile phosphate buffer solution to remove any non-adherent bacteria. The samples were then incubated at 37oC for 48 hours. The samples were then rolled onto sterile blood agar under sterile precautions. These plates were further incubated at 37oC for 48 hours. Colony counting was done for each group and results were compared with control of each batch. In case of any contamination, the whole batch was rejected and the procedure was repeated. The data was collected and statistical analysis was done.

Results: The maximum reduction in the number of CFU and adherence is observed with Group G: Laser irradiation 3.5 watts for 30 sec followed by application of remineralizing paste CPP ACP F. Thereafter the next best results were with Group H, Group F, Group B. Group E: IgY

Conclusions: Laser irradiation with or without pastes brings about a reduction in CFU and adherence of streptococci mutans. Thereafter the non-laser irradiation protocol which shows a reduction in the bacterial count is IgY group.

Keywords: Dental caries, streptococci mutans, Laser, adherence, IgY

Introduction

The oral cavity is the mirror to the health of a human being. According to Loesche, the oral cavity is the abode of six billion microorganisms.[1] The oral ecological niche is a haven for microorganism. The tooth surface is coated with a thin layer of pellicle as soon as it is cleaned. This is the initiation of biofilm formation. The unique property of the tooth surface which makes it an ideal ecological niche is its inability to shed the surface layer, thus the biofilm which is formed cannot be dislodged easily and has to be mechanically removed.[2]

Mutans streptococci are an established pioneer cariogenic microbe, owing to their inherent characteristics of being acidogenic and aciduric. Thus, they produce short chains of acid which causes subsurface demineralization. The tooth surface as soon as it is cleaned, develops a layer of acquired pellicle on the enamel. Streptococci mutans participate in the formation of biofilms on the dental hard tissues.[2] Streptococci mutan's adhesion within the biofilm can be determined by a sucrose dependent or sucrose-independent pathways. The sucrose-free/ independent adhesion to salivary galactoside within the acquired enamel pellicle commences the attachment undertaking, in contrast, sucrose-dependent adhesion is fundamentally liable for the colonization on dental hard tissue surfaces and also for orchestrating the change in an ecological milieu which will eventually lead to the formation of dental caries.[3] Sucrose is essential for the mutan streptococci to adhere and accumulate on the tooth. These cariogenic bacteria adhere by streptococcal adhesins, known as Antigen I & II, to the galactoside of the saliva derived glycoprotein of the enamel pellicle. Antigen I & II are also known as streptococcal protein antigen P, which has been implicated in the formation of dental caries owing to its virulence.[4] The other surface moieties of streptococci mutans for adherence include the enzymes glucosyltransferases (GTFs), which are inherently produced and synthesized by the cariogenic mutans, the other moiety is serotype carbohydrate and glucan-binding protein (GBP). These isozymes of glucosyltransferases are responsible for catalysing and metabolizing sucrose with resultant formation of extracellular polysaccharides which increase the

adherence of streptococci to hard tissues of the tooth. Within the plaque, the adhesion of mutans streptococci is brought about by both sucrose dependent mediation and sucrose independent mechanism. Saliva derived components within the acquired enamel pellicle may be responsible for the primary adhesion in sucrose independent pathway whereas, sucrose is primarily responsible for agglomeration and colonization on the dental hard tissue surface. [4] In the omnipresence of sucrose, the cell-wall-associated glucosyltransferases transfigure the extracellular sucrose into glucan.[4] This in association with glucan-binding proteins (GBP) located on the surface of the bacteria, facilitate cell to cell aggregation. [4,5]Streptococci mutans generates the following three types of glucosyltransferases i.e GTFB, GTFC, GTFD, whose cumulative effort is crucial for adherence of microbial cells. There has been the identification of four various types of Glucan binding proteins (GBP): GBP-A, GBP-B, GBP-C and GBP-D. out of which GBP-A and GBP-C have shown a direct correlation and attributes to the cariogenicity of streptococci mutans.[6,7] In the absenteeism of sucrose, the adhesion of Streptococci mutans to the hard tissue of the tooth, or with other microorganisms in oral film, is conciliated by several surface moieties of adhesins.[4,5] Streptococcus mutans is competent in agglomerating in the oral cavity and adhering to dental hard tissues and thus leading to the formation and maturation of microbial biofilm. In addition to its characteristic of colonization and coaggregation, additional properties enabling S. mutans to survive in an acidic oral milieu and have specific interactions with other microbial species inhabiting the oral environment.[4-8]

Hence various modalities of reducing the adherence of streptococci mutants and thus reducing the microbial load which can initiate dental caries need to be studied.

Hence, this study aims to evaluate different surface treatment protocols efficacy in reducing the adherence of streptococci mutans to enamel.

Material & Method

1. Aluminium Gallium Arsenide Laser (WhitestarTM, Creation, Verona, Italy)
2. Acidulated phosphate fluoride (APF) gels 1.23% (Pascal USA)
3. Casein Phosphopeptide -Amorphous Calcium Phosphate Fluoride (CPP-ACPF) paste (GC Tooth Mousse)
4. 37 % phosphoric acid gel (Total Etch™– Ivoclar Vivadent AG, Schaan /Liechtenstein)
5. Ig Y tablets (Nodecay – Tata Inzpera)
6. ATCC strain of Streptococcus mutans (25175)

Procedure

Waiver for informed consent was taken. 104 freshly extracted molars were collected. After disinfection, the occlusal surface were decoronated with a diamond disc. The occlusal surface samples were randomly allocated to eight groups which are the following:Group A: Control - No surface treatment

- Group B: Laser irradiation of 3.5 watts of 810nm of Aluminium Gallium Arsenide laser for 30 sec.
- Group C: 1.23 APF gel
- Group D: Re-mineralizing paste (CPP ACP F)
- Group E: IgY
- Group F: Laser irradiation 3.5 watts for 30 sec followed by fluoride treatment of 1.23 APF gel
- Group G: Laser irradiation 3.5 watts for 30 sec followed by application of re-mineralizing paste CPP ACP F
- Group H: Laser irradiation of 3.5 watts followed by treatment with IgY

ATCC strain of Streptococcus mutans (25175) were procured (HI Media Laboratories Pvt. Limited) and sub-

cultured as per standard guidelines. The purity of cultures was confirmed by staining and biochemical reactions. The organism was then sub-cultured in Brain Heart Infusion broth (BHI) and incubated at 37°C to adjust the suspension to 0.5 Mac Farland standard, which corresponds to 10^8 colony-forming units per millilitre (CFU/ml). The enamel samples were surface treated as appended above. For group including IgY, the tables were made to dissolve in normal saline so that a thick paste was available for coating the occlusal surface of the samples. The teeth samples were immersed in the broth in separate sterile containers and agitated at 80 rpm for 90 minutes to promote adhesion of the organism on tooth surface. Following this, the broth was aspirated and teeth samples were washed twice with sterile phosphate buffer solution to remove any non-adherent bacteria. The samples were then incubated at 37°C for 48 hours. The samples were then rolled onto sterile blood agar under sterile precautions. These plates were further incubated at 37°C for 48 hours. Colony counting was done for each group and results were compared with control of each batch. In case of any contamination, the whole batch was rejected and the procedure was repeated. The data was collected and statistical analysis was done.

Results

Using SPSS 24 version, One-way analysis of variance (ANOVA) was used to determine whether there were statistically significant differences between the means of three or more independent groups. Since the p-value was less 0.05, hence a post hoc multiple comparison Tukey's was used to test differences among sample means for significance. There was high variation in data and it was heterogeneous, so the non-parametric test was applied for analysis. The Kruskal-Wallis H test, a rank-based nonparametric test was used to determine if there are statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable. Kruskal-Wallis Test was applied to compare values between the groups followed by Mann-Whitney-U test for multiple comparisons. A p-value less than 0.05 considered as significant at 95% confidence level.

The control group had the greatest number of the colony-forming unit. The laser irradiation of 3.5 watts of Aluminium Gallium Arsenide laser for 30 sec on enamel surface followed by CPP ACP F yielded the least nos of colony-forming units. Even when the laser-irradiated was followed by application of IG- Y paste, APF gel it yielded comparable result. The next best result was of laser irradiation. These results were statistically significant. The next best results, in terms of reduction in colony-forming units, were seen with IG- Y and the worst results were seen in untreated control samples. (Table, Graph 1 and Graph 2)

Discussion

Dental caries is a predominant oral disease with a far-reaching ramification. Cariology research has proved that dental caries is a sequela of the transmutation of a healthy non-cariogenic oral microbiome into an acidogenic cariogenic microbiome.[9,10] In 2008, the National Institute of Health introduced the (HMP) Human Microbiome Project identifying and acknowledging the existence of the human microbiome and evidence-based studies validating it. Various microbial studies and newer molecular identification studies and metabolic analysis have all reiterated the importance of deciphering the cariogenic oral microbiome.[11,12]

Various modalities have been discussed to challenge the oral microbiome to prevent and inhibit dental caries. Various authors have suggested various variegated method to modify the ecological niche of streptococci mutans.[13-15]' Since dental caries is not a classical infectious disease

but a sequela of ecological shift, hence various studies have tried to correlate diet and microbial mediators of the cariogenic onslaught.[13-16] Fluorides is no longer the dernier cri of cariology. Studies have shown that repeated topical application of fluoride will give rise to fluoride resistant strains of streptococci mutans.[17] Currently suggested modalities have suggested looking beyond the fluoride shibboleth.[18,19] A wide range of modalities have been reviewed in literature from use of, CPP ACP, novamin, immunogens, bacteriophages and nanotechnology, specific target studies, all these treatment options targeted the reengineering the dysbiosis of the oral microbiome.[14-16,18-21]

One technique to correct the cariogenic dysbiosis is to alter or biomodification of enamel surface by the use of lasers which in turn will increase the acid resistance of the tooth. Sharma et al have carried a series of studies and it was observed that aluminium gallium arsenide laser brought about an increase in hardness of irradiated enamel surfaces. It has been postulated that after laser irradiation there is greater increased acid resistance and the uptake of the fluoride is increased. [22-26] Hence in this study the same protocol was replicated and it was observed that laser, when used with CPP ACP F, gave the best results, or when used alone or in combination with other pastes like fluoride or Ig -Y, it gave comparable results and brought about a decrease in adherence and it was statistically significant. The next best statistically significant result was with the Ig-Y application group. The other pastes when used alone showed a decline in bacterial load but not as the laser and paste combination groups or laser irradiation group. (Table, Graph 1 and Graph 2)

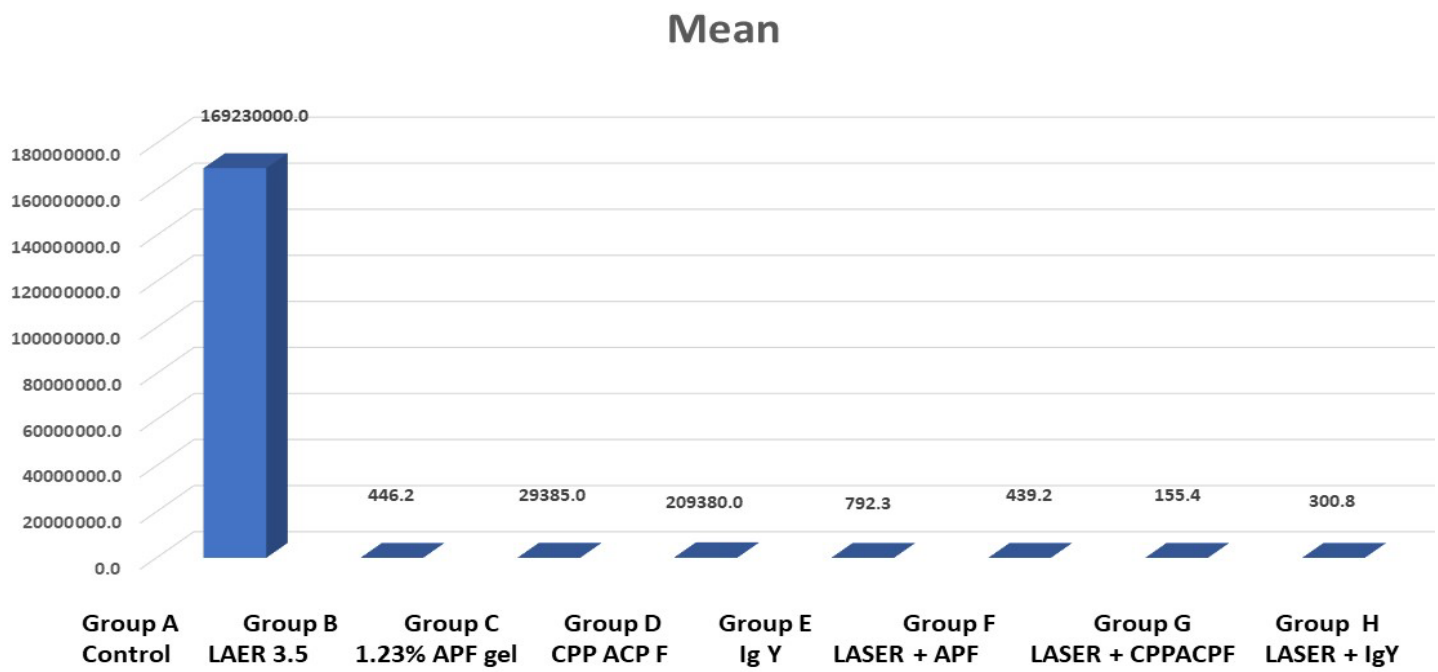
Various surface treatment protocol has been evaluated by Sharma et al to inhibit dental caries. The surface treatment protocols which were evaluated were CPP ACP F, Enafix used alone on in combination with different wattages of lasers to remineralized demineralized enamel surface.[22-26] Hence, in this study, we have evaluated fluoride APF gel and CPP ACP F and a paste of Ig-Y. It was observed that out of the pastes the Ig – Y performed better than that of both APF gel and C PP ACP F. 1.23 % APF gel performed better in reducing streptococcal load than CPP ACP F group.

Table: Multiple Comparisons Of The Different Surface Treatment Protocols of Enamel Surface.

Mann-Whitney Test							
Test Statistics	P value						
p-value	Group B Laser 3.5w	Group C. APF Gel 1.23%	Group D CPP/ACPF	Group E Ig Y	Group F Laser + APF	Group G Laser + CPP ACPF	Group H Laser + Igy
Group A. Control	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Group B. Laser 3.5w		<0.001	<0.001	0.052	0.813	0.031	0.295
Group C. APF Gel 1.23%			0.05	<0.001	<0.001	<0.001	<0.001
Group D. CPP ACP F				<0.001	<0.001	<0.001	<0.001
Group E. Ig Y					0.044	<0.001	0.006
Group F Laser + Apf						0.088	0.474
Group G. Laser + CPP ACP F							0.263
Group H. Laser + Igy							

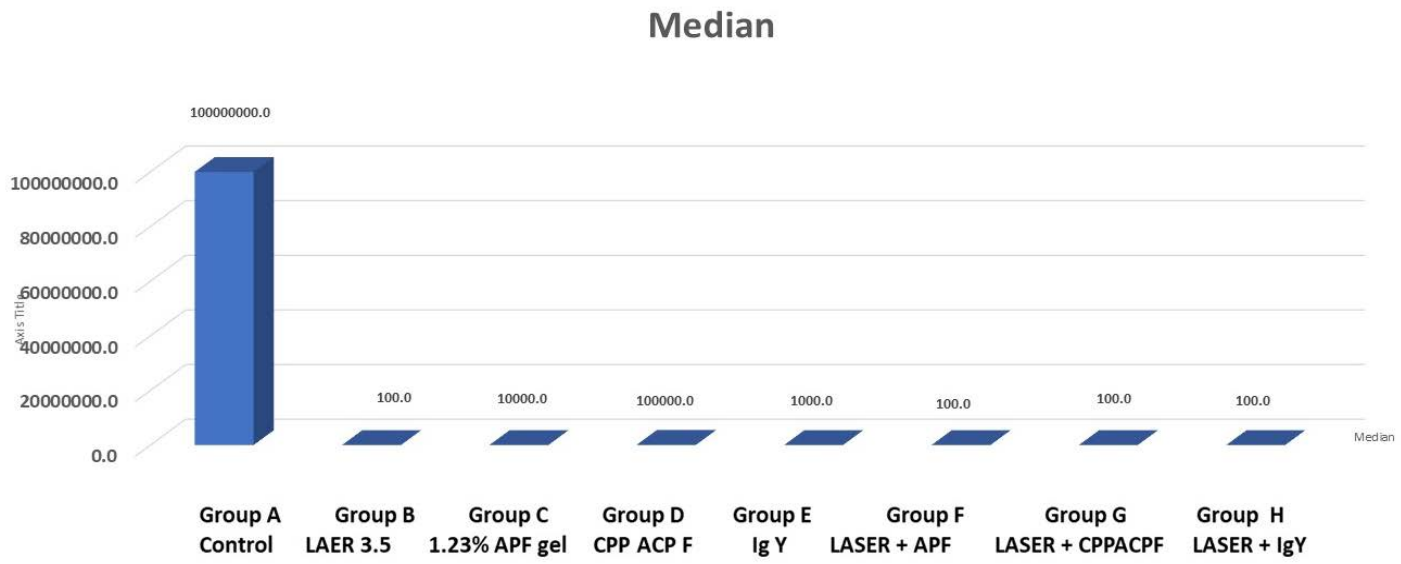
The test was applied to see the difference between the different groups for non-normal data. All the groups have significant difference (Median) with each other at 95% confidence level except for the few comparisons which are as follows:Group B is non-significantly different (or similar) with group E, F and H Group F is non-significantly different (or similar) with group G and H and group G is similar to group H.

Graph 1: Comparison And Correlation of Effect of Different Surface Treatment Protocols on Adherence of Streptococci Mutans to Enamel : Mean



On comparing the mean of all samples it was observed:All treatment protocols when compared to control did reduce the streptococci mutans cariogenic load and its adherence. 3.5 watts aluminium gallium arsenide laser irradiation for 30 secs followed by CPP ACP F paste application group, could reduce the adherence of the streptococci significantly. The next group in terms of efficacy was Laser followed by IgY application, this was followed by Laser and APF gel. Next efficacious treatment protocol was irradiating the enamel samples with 3.5 watts of aluminium gallium arsenide laser irradiation for 30 secs. Igy group when used alone gave a better score than it counterparts of group C and group D .

Graph 2: Comparison and correlation of Effect of different surface treatment protocols on adherence of streptococci mutans to enamel : Median



On comparing the medians of all the samples it was observed:

All treatment protocols when compared to control did reduce the streptococci mutans cariogenic load and its adherence. All the groups which had laser irradiation and in combination with pastes gave comparable results. IgYgroup when used alone gave a better score than it counterparts of group C and group D

In vitro studies and rat model studies done by Gandimati [27] and Sentila [28] have revealed that fowl egg yolk antibodies against Cell Associated Glucosyltransferase can be employed as an efficacious caries preventive modality. A rat model study evaluated the short term (a week to 3 weeks) effect of oral passive immunization on the cariogenic potential of streptococci mutans. It was observed that passive antibody to S. mutans glucan binding protein B (GBP-B) -B has caries preventive effect.[29] Similarly in this study, it was observed that IgY group reduced the streptococcal load in the samples significantly but fared poorer than Laser paste combination groups and Laser irradiation groups, but it fared significantly better than the 1.23 % APF gel group and CPP ACP F paste. (Graph 1 & 2)

Conclusion

In this adherence study comparing different surface treatment protocols efficacy in reducing the adherence of streptococci mutans on enamel samples, it can be concluded within the limitations of the study that:

1. Aluminium gallium arsenide laser 3.5 watts irradiation for 30 secs followed by CPP ACP F paste application group, could reduce the adherence of the streptococci significantly. The next group in terms of reduction of the colony-forming unit was observed in the Laser followed by IgY application group, this was followed by Laser and APF gel. Next efficacious treatment protocol was irradiating the enamel samples with 3.5 watts of aluminium gallium arsenide laser irradiation for 30 secs.
2. All treatment protocols did reduce the streptococci mutans cariogenic load and its adherence. However, the inclusion of lasers did bring about a reduction in microbial load. Hence long term clinical trials need to be undertaken.

3. Passive immunization with Ig Y also reduced the adherence and fared better than surface treatment protocols which included the remineralization pastes. Hence, passive immunization can also be researched further for caries prevention.

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