Original Research Article

Effect of Immunoglobulin Y tablets on streptococcus mutans counts in adolescents: An in vivo study

ABSTRACT

Objectives: Streptococcus mutans has been most intimately associated with the initiation and development of carious lesion. Nowadays, the treatment strategies are aimed at either elimination of this bacterium or suppression of its virulence. The search for more efficient and economical techniques, has led to growing interest for the use of egg yolk antibodies (Immunoglobulin Y). This study was conducted to evaluate the microbiological effect of Ig Y tablets on salivary Streptococcus mutans count in adolescents.

Methods: In this randomized controlled clinical trial, 50 adolescents (12–18 years old) were divided into two groups. Group I adolescents were instructed to use fluoridated toothpaste and Group II were instructed to administer S. Mutans specific Immunoglobulin Y chewable tablets for a period of 15 days along with the fluoridated toothpaste.

Results: The findings of this research demonstrated that the tablets containing specific egg yolk antibodies (IgY) against glucosyltransferase of S. mutans exerted a suppressive effect on S. mutans colonization of the oral cavity in healthy adolescents.

Conclusion: These findings indicate that using IgY in the form of tablets is a novel approach and has the advantage of releasing antibodies slowly into the oral cavity. It is thus effective in decreasing the colonization of the S. mutans, and lowering the induction of dental caries.

Keywords: Immunoglobulin Y; Streptococcus Mutans; Glucosyltransferase; Ag I/II

INTRODUCTION

"Dental caries manifests as a continuum of disease states of increasing severity and tooth destruction, ranging from subclinical changes to lesions with dentinal involvement".[1] According to the data from the systemic analysis "The Global Burden of oral conditions (2021)"[2], the untreated caries in permanent dentition was believed to be the most prevalent dental disease (29.4%), affecting 2.5 billion people worldwide.[3] "Prevention of this polymicrobial disease has been the area of research since the beginning. Several preventive strategies are being used for the prevention and control of caries which involves disorganization of the biofilm through mechanical and chemical plaque removal measures in addition to use of fluorides. However, the still high prevalence of such disease in the world population, justifies the search for new preventive actions, amongst

which the immunization of the dental caries is the emerging approach. Immunization against dental caries aims to decrease the count of the cariogenic species and is an innovative way to undertake prevention programs".[4]

A variety of acidogenic bacterium have been known to be associated with different stages of dental caries, however, S. mutans is implied to be the leading causative microorganism.[14] "It is also considered as the most cariogenic among all genera of oral streptococci. Streptococcus mutans has three main groups of virulence factors that allow it to adhere and accumulate in dental biofilm. These antigens are the main targets for the development of the caries vaccine, and include; glucosyltransferases, antigenic adhesives I / II glucan-binding proteins. Thus, for immunization, the main target is the mechanism of adherence of S. mutans, which can be affected by either active or passive immunization, or by DNA vaccines".[4]

Antibodies against antigens from S. mutans have been found to be present in the gingival crevicular fluid and saliva in the immunized hosts. These antibodies lead to the formation of long chains and fine meshed colonies of bacteria in the oral cavity. These have reduced pathogenic potential and can be easily removed from the oral cavity.[5] Moreover, the epitopes of the adhesins of S. mutans might be blocked by these antibodies.

Experimental immunization against S. mutans brings about high levels of specific salivary antibodies, correlating with prophylaxis against caries development. Countless efforts have been made to improve the immune response against the bacteria using different adjuvants and delivery vehicles and through various routes of vaccination like subcutaneous, oral, intranasal, and topical routes. They have all been able to give rise to both mucosal and systemic response and reduce the S. mutans in plaque and thus lessen the development of dental caries.[6] In the initial studies, a whole S. mutans bacterium was utilized, but the antibodies generated were found to be cross-reactive against heart muscle tissue.[7] "Due to this danger, the development of subunit vaccines has been the centre of interest. However, these caries vaccines are costly and considered to be effective only in animal models, and are not available commercially for human use".

Considering the stumbling blocks involved in caries vaccine, passive immunization has gained attention in the recent past. The passive immunization is achieved by the transfer of prefabricated antibodies, which circulate in the body and give specific protection.[4] Experimentally successful passive vaccines for caries have been delivered in many forms, including monoclonal mice IgG antibodies, bovine or ape IgG antibodies and chicken IgY. Recently, local passive immunization using Egg-yolk antibodies, (Ig Y) to antigens of S. mutans has gained attention.[6] "This IgY inhibits caries development by binding to S. mutans antigens and neutralizing the biological activity of the antigen".[8] "It is usually complemented with regular oral hygiene practices for decreasing the percentage of S. mutans in human saliva".[9]

"Numerous *in vitro* and animal studies have shown that IgY specific to glucosyltransferase showed a statistically significant reduction in dental plaque accumulation and caries development".[10] However, there is little evidence that IgY could affect the level or cariogenic properties of S. mutans in vivo.

In this research, the effect of Immunoglobulin Y was evaluated by estimating the S. mutans count in saliva, salivary pH and plaque pH pre and post administration of the IgY tablets at 15 days, 30 days and 60 days interval.

Subjects and Methods

The present research was undertaken as a prospective, parallel group, randomized controlled, open label study conducted in the post graduate dental college for a period of 6 months.

The study design included a screening using caries risk assessment form, a saliva sample, and a plaque pH evaluation at baseline, after 15 days, 30 days and 60 days after the administration of IgY tablets for 15 days.

Treatment and Sample Collection

Generation of the random sequence of the study was done by computer generated randomization. According to power analysis, the sample size was calculated to be 25 for each group i.e. cases and controls at p-value of 0.05. Hence, the total sample size of the study was set as 50 with cases and controls. Group I consisted of 25 children and was instructed to use a fluoridated toothpaste, Colgate® Total 1000 ppm twice daily at least for the trial duration, i.e., 2 months. 25 Children falling under Group II were instructed to administer S. mutans specific Immunoglobulin Y chewable tablets (No DecayTM Chewable Tablet, Inzpera Health Sciences) for a period of 15 days. Children were asked to chew one 20 mg chewable tablet (orange in colour) in the morning after breakfast and one 40 mg chewable tablet (white in colour) after dinner before going to sleep. Along with the tablet, children were asked to use Colgate® Total 1000 ppm twice daily for a minimum of 2 months. Children were advised to brush their teeth before chewing the tablet and not eat or drink for at least one hour after chewing the tablet.

Plaque Collection and Analysis

Plaque specimens were collected by scraping the surfaces of the molars using a sterile spoon excavator. 2 ml of triple deionized water was measured using a graduated pipette and poured into a clean beaker. The plaque sample collected was then dissolved in the water and pH measured using pH meter.

Saliva Analysis

Determination of plaque pH

A digital pH meter (Hanna instruments, Italy) was used to assess the pH of the collected unstimulated and stimulated saliva. In between the readings, the pH meter was cleaned with a stream of distilled water and placed in a standard solution of 7.0 which ensured a stable reading. The pH was determined as soon as possible, but no later than 30 minutes. ***Microbial analysis***

An analysis of the MS concentration in saliva was performed. For each patient, both the stimulated and unstimulated saliva collection was carried out. The samples were transported to the Department of

Microbiology and processed within 45 min after collection. The samples were serially diluted in sterile phosphate buffered solution. Aliquots of 0.5 mL were inoculated on mitis-salivarius bacitracin agar. The plates were incubated in a 5% CO 2 environment at 37° C for 72 h after which the colony-forming units were counted manually with the help of magnifying lens. The colonies of S. mutans were identified as round or spherical, raised, convex, black in colour, ranging from a pinpoint to pinhead size with a rough surface. The colony count of the agar plate was recorded and the mean CFUs per millilitre were determined after multiplying the colony count of each plate with its respective dilution factor.

Statistical Analysis

The IBM SPSS software program version 20.0 was used to examine the data that was supplied into the computer. Range (minimum and maximum), mean, standard deviation, and standard error of mean were used to characterize quantitative data. The data was graphically represented using a bar chart. The Kolmogorov–Smirnov test was used to check the normality of the quantitative variable distributions. The data were found to be regularly distributed, hence the parametric test was used. An independent t test was used to compare two independent populations, while the paired t test was utilized to assess two paired data. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

This study's findings were collected from 50 adolescents ranging in age from 12 to 18 years old. They were easily chosen based on the criterion for inclusion. A group of patients was chosen and separated into two groups (study group and control group).

TABLE 1 : GENDER DISTRIBUTION IN THE TWO GROUPS

Gender	Group 1		Group 2		Total
	N	%	N	%	
Female	15	60.0	14	56.0	29
Male	10	40.0	11	44.0	21
Total	25	100.0	25	100.0	50

x^2 value = 0.082; df = 1; p = 0.774; Not significant

The gender distribution of the analyzed sample groups is shown in Table 1. The control group consisted of 10 males and 15 females, with a percentage of 40 and 60, respectively. The study group consisted of 11 males and 14 females, with a percentage of 44 and 56, accordingly. With a p value of 0.774, there was no statistically significant difference between the mean genders in the two groups. **STREPTOCOCCUS MUTANS COUNT IN UNSTIMULATED SALIVA**

TABLE 2: INTER- GROUP COMPARISON OF S. MUTANS COUNT IN UNSTIMULATED SALIVA AT DIFFERENT TIME INTERVALS

Time	Group 1 (n = 25)			Group 2 (n = 25)			't' value	P value
	Mean	± SD	SEM	Mean	± SD	SEM		
Baseline	7.899	2.066	0.41	7.626	1.978	0.39	0.477	0.635; NS
After 15 days	7.546	1.985	0.40	6.720	1.804	0.36	1.539	0.130; NS
After 30 days	7.239	1.900	0.38	5.778	1.583	0.32	2.954	0.005*
After 60 days	6.860	1.829	0.37	4.694	1.353	0.27	4.757	<0.001**

NS: p > 0.05; Not Significant; *p<0.05; Significant; **p<0.001; Highly significant

TABLE 3: INTER- GROUP COMPARISON OF S. MUTANS COUNT IN STIMULATED SALIVA AT DIFFERENT TIME INTERVALS

Time	Group 1 (n = 25)			Group 2 (n = 25)			't' value	P value
	Mean	± SD	SME	Mean	± SD	SME		
Baseline	7.663	1.887	0.38	7.182	1.718	0.34	0.943	0.351; NS
After 15 days	7.178	1.852	0.37	6.463	1.748	0.35	1.402	0.167; NS
After 30 days	6.774	1.850	0.37	5.489	1.432	0.28	2.745	0.008*
After 60 days	6.373	1.758	0.35	4.528	1.222	0.24	4.308	<0.001**

NS: p > 0.05; Not Significant; *p<0.05; Significant; **p<0.001; Highly significant

Table 2 and Table 3 show the inter group comparison of S. Mutans count in unstimulated and stimulated saliva respectively at different time intervals. It reveals that at the baseline, the difference in the mean bacterial count between the two groups was not statistically significant (p > 0.05) but a significant change in count was observed on subsequent intervals. The percent reduction in S. mutans count was observed to be 11.8%, 27.5% and 50.7% at 15th, 30th and 60th day intervals respectively in the unstimulated saliva. The percent reduction in S. mutans count was observed to be 9.97%, 27.5% and 48.33% at 15th, 30th and 60th day intervals respectively in the stimulated saliva group. On intergroup comparison,

statistically significant difference was observed at 30th day and a highly significant difference was observed at 60th day interval.

CHANGE IN SALIVARY pH IN UNSTIMULATED SALIVA

TABLE 4: INTER- GROUP COMPARISON OF pH OF UNSTIMULATED SALIVA AT DIFFERENT TIME INTERVALS

Time	Group 1 (n = 25)			Group 2 (n = 25)			't' value	P value
	Mean	± SD	SME	Mean	± SD	SME		
Baseline	6.500	0.464	0.09	6.436	0.408	0.08	0.514	0.610; NS
After 15 days	6.650	0.448	0.08	6.588	0.413	0.08	0.512	0.611; NS
After 30 days	6.737	0.438	0.08	6.794	0.405	0.07	0.482	0.632; NS
After 60 days	6.818	0.431	0.08	7.080	0.382	0.07	2.274	0.027*

NS: p > 0.05; Not Significant; *p<0.05; Significant; **p<0.001; Highly significant

Table 4 shows the inter- group comparison of pH of unstimulated saliva at different time intervals. At baseline, the mean salivary pH in Group I ranged from 6.5 ± 0.464 and in group II, it ranged from 6.4 ± 0.408. When a t-test was performed, the p – value was found to be 0.610, which infers that the difference between the mean salivary pH within two groups was insignificant. At subsequent intervals, an overall increase in pH was observed in both the groups with a statistically highly significant difference in the two groups at 60th day interval.

CHANGE IN SALIVARY pH IN STIMULATED SALIVA

TABLE 5: INTER- GROUP COMPARISON OF pH OF STIMULATED SALIVA AT DIFFERENT TIME INTERVALS

Time	Group 1 (n = 25)			Group 2 (n = 25)			't' value	P value
	Mean	± SD	SME	Mean	± SD	SME		
Baseline	6.596	0.466	0.09	6.502	0.408	0.08	0.758	0.452; NS
After 15 days	6.716	0.444	0.09	6.631	0.417	0.08	0.695	0.490; NS
After 30 days	6.788	0.437	0.08	6.788	0.405	0.08	0.473	0.639; NS
After 60 days	6.882	0.467	0.09	7.139	0.384	0.07	2.130	0.038*

NS: p > 0.05; Not Significant; *p<0.05; Significant; **p<0.001; Highly significant

Table 5 shows the inter- group comparison of pH of stimulated saliva at different time intervals. At baseline, the mean salivary pH in Group I ranged from 6.59 ± 0.466 and in group II, it ranged from 6.50 ± 0.408. When a t- test was performed, the p – value was found to be 0.758, which infers that the difference between the mean salivary pH within two groups was insignificant. On subsequent intervals, an overall increase in pH was observed in both the groups with a statistically significant difference in Group II at 60th day interval.

PLAQUE ACIDOGENICITY

TABLE 6: INTER- GROUP COMPARISON OF pH PLAQUE AT DIFFERENT TIME INTERVALS

Time	Group 1 (n = 25)			Group 2 (n = 25)			't' value	P value
	Mean	± SD	SME	Mean	± SD	SME		
Baseline	6.539	0.507	0.10	6.485	0.441	0.09	0.408	0.685; NS
After 15 days	6.624	0.504	0.10	6.646	0.432	0.09	0.169	0.867; NS
After 30 days	6.710	0.508	0.10	6.839	0.436	0.08	0.962	0.341; NS
After 60 days	6.800	0.510	0.10	7.063	0.437	0.08	1.956	0.056; NS

NS: p > 0.05; Not Significant; *p<0.05; Significant; **p<0.001; Highly significant

Table 6 shows inter-group comparison of pH plaque at different time intervals. Changes in plaque pH were observed at four different time intervals. At baseline, the two groups resulted in a similar pH pattern, while an

increase in pH was observed on day 15, 30 and 60. The difference between the two groups was statistically insignificant on intergroup comparison.

DISCUSSION

In the present study, the effect of Immunoglobulin Y was evaluated by estimating the S. mutans count in saliva, salivary pH, and plaque pH pre and post administration of the IgY tablets at 15 days, 30 days and 60 days intervals. The first parameter observed in the study was the salivary levels of S. mutans. The results demonstrated a significant reduction in the count in both the groups. The overall mean bacterial count decreased from 7.626 ± 1.978 to 4.694 ± 1.353 in the two month period in the study group. This reduction in the S. mutans count in the IgY group can be attributed to the protective effect of passive immunization. The microorganisms present in the saliva could be eliminated from the oral cavity through passive immunization by antibody-mediated accumulation, before these organisms are able to colonize the teeth. The cell wall associated glucosyltransferase of S. mutans plays a vital role in pathogenicity of dental caries by conversion of sucrose to long – chain polysaccharides.11 The IgY acting against this cell wall associated glucosyltransferase inhibits the adherence of S. mutans to the tooth surface and interferes with the glucosyltransferase activity thus inhibiting the initiation of dental caries.10 This passive immunization route have succeeded in decreasing the number of S. mutans count and thereby lowering the caries development in the animal experiments as done by Koga T et al and Krüger, C et al 12,13 which showed that the antiglucosyltransferase group developed lower and less extensive dentinal caries than the control group.

The second parameter observed in the study was the salivary pH which is an important salivary parameter that affects the carious process. A baseline, the difference between the mean salivary pH within two groups was insignificant. At subsequent intervals, an overall increase in pH was observed in both the groups with a statistically highly significant difference in the two groups at 60th day interval. According to, Banas JA in 201480 the acidification of the external environment is a consequence of the excretion of acid by products of bacterial metabolism. Hence, a significant rise in salivary pH in our study could be due to the reduction in S. mutans. Our results were in accordance with the study done by Fahad AH et al, where a negative correlation was recorded between salivary streptococcus mutans count with salivary pH in adolescents.

In the present study, changes in plaque pH were also observed at four different time intervals. At baseline, the two groups resulted in a similar pH pattern, while an increase in pH was observed on day 15th, 30th and 60th day. The difference between the two groups was statistically insignificant on intergroup comparison. On intra group comparison significant reduction in plaque acidogenicity was observed within both the groups with a highly significant change in the IgY group.

The IgY antibodies may interfere with the initial stage of dental plaque formation by means of specific mechanisms i.e., either by aggregation of the bacteria before colonization or by blockage of surface receptors necessary for colonization. In our study, the Ig Y might have affected S. mutans colonization at all the three points of bacterial vulnerability. First, the dimeric Fab domain of IgY might have made the bacterial cells

susceptible to displacement by salivary movement by aggregation of these target bacterial cells into larger clusters and rendering them unable to adhere and colonize tooth surfaces. Secondly, the glucosyltransferase catalytic site might have been abolished by the polyclonal IgY antibodies. This might affect the glucan-dependent adherence of S. mutans thus rendering them unable to attach to the tooth surfaces or might also affect the already adhered S. mutans to initiate plaque deposition or to build on existing dental plaque. This view is supported by the results of animal experiments in rats using glucosyltransferase-defective,84 or glucosyltransferase-negative83 S. mutans that failed to colonize the oral cavity. Third, the natural glucan-binding domains present at the carboxylterminal portion of glucosyltransferase might have been blocked. This inhibits the adherence of S. mutans cells onto existing dental plaques and thereby preventing the supercolonization of S. mutans in the oral cavity. Thus, the suppressive effect of anti glucosyltranferase IgY appears to strike at various stages of dental plaque formation. This decrease in colonization of S. mutans leads to reduction in the plaque acidogenicity.

Taken together, in the present study we evaluated the concept that tablets containing specific egg yolk antibodies (IgY) against glucosyltransferase of S. mutans exerted a suppressive effect on S. mutans colonization of the oral cavity in healthy adolescents

CONCLUSION

In conclusion, these results suggest that tablets containing anti glucosyltransferase IgY when complemented with regular oral hygiene practices can reduce the S. mutans count effectively. These tablets can be taken safely as none of the participants in our study reported any adverse events in their overall health condition during the trial and two weeks after the trial ended. Children in our study exhibited more acceptance and compliance due to the favourable taste and odour of the tablets. The use of IgY in the form of tablet is a novel approach and has the advantage of delivering antibodies into the oral cavity slowly.

DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Ethical Approval:

The present study was prospectively approved by the Institutional Research & Ethical Committee and was registered with the Clinical Trial Registry- India (CTRI number – CTRI/2020/05/025447) under ICMR – National Institute of Medical Statistics.

References

1. Featherstone JD. The continuum of dental caries—evidence for a dynamic disease process. Journal of dental research. 2004 Jul;83(1_suppl):39-42.
2. Marcenes W, Bernabé E. Global Burden of Oral Conditions. InOral Epidemiology 2021 (pp. 23-37). Springer, Cham.
3. BD 2017 Oral Disorders Collaborators, Bernabe E, Marcenes W, Hernandez CR, Bailey J, Abreu LG, Alipour V, Amini S, Arabloo J, Arefi Z, Arora A. Global, regional, and national levels and trends in burden of oral conditions from 1990 to 2017: a systematic analysis for the global burden of disease 2017 study. Journal of dental research. 2020 Apr;99(4):362-73.
4. Tello PM, Tamayo GC, Ortiz HA, Coyago MD. Immunization strategies in dental caries: Scopes and limitations. World Journal of Advanced Research and Reviews. 2020;8(1):076-85
5. Liljemark WF, Bloomquist CG, Germaine GR. Effect of bacterial aggregation on the adherence of oral streptococci to hydroxyapatite. Infection and immunity. 1981 Mar;31(3):935-41.
6. Koga T, Oho T, Shimazaki Y, Nakano Y. Immunization against dental caries. Vaccine. 2002 May 15;20(16):2027-44.
7. Hughes M, Machardy SM, Sheppard AJ, Woods NC. Evidence for an immunological relationship between Streptococcus mutans and human cardiac tissue. Infection and immunity. 1980 Feb;27(2):576-88.
8. Bachtiar EW, Apritantia DE, Sarwono AT, Bachtiar BM, Soejoedono RD, Wibawan WT. The Effect of Mouthwash Combination of Immunoglobulin-y Anti-Comd Streptococcus Mutans and Chitosan on the Formation of Streptococcus Mutans Biofilm. International Journal of Applied Pharmaceutics. 2019 Apr 1;11(4):110-3.
9. Gani BA, Chismirina S, Hayati Z, Winiati E, Bachtiar BM, Wibawan IW. The ability of IgY to recognize surface proteins of Streptococcus mutans. Dental Journal (Majalah Kedokteran Gigi). 2009 Dec 1;42(4):189-93.
10. Hatta H, Tsuda K, Ozeki M, Kim M, Yamamoto T, Otake S, Hirasawa M, Katz J, Childers NK, Michalek SM. Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to Streptococcus mutans. Caries research. 1997; 31(4):268-74.
11. Abiko Y. Passive immunization against dental caries and periodontal disease: development of recombinant and human monoclonal antibodies. Critical Reviews in Oral Biology & Medicine. 2000 Apr; 11(2):140-58
12. Koga T, Oho T, Shimazaki Y, Nakano Y. Immunization against dental caries. Vaccine. 2002 May 15;20(16):2027-44.
13. Krüger C, Pearson SK, Kodama Y, Smith AV, Bowen WH, Hammarström L. The effects of egg-derived antibodies to glucosyltransferases on dental caries in rats. Caries research. 2004;38(1):9-14.
14. Cherukuri G, Veeramachaneni C, Rao GV, Pacha VB, Balla SB. Insight into status of dental caries vaccination: A review. Journal of Conservative Dentistry: JCD. 2020 Nov;23(6):544.