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**EFFICACY OF ORAL PASSIVE IMMUNOTHERAPY AGAINST DENTAL
CARIES IN HUMANS USING CHICKEN EGG YOLK ANTIBODIES
GENERATED AGAINST *STREPTOCOCCUS MUTANS***

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ABSTRACT

Dental caries an ever growing public health problem is not much amenable to existing preventive measures. The aim of this study was to compare and evaluate the effectiveness of mouth rinse containing chicken egg yolk antibodies generated against whole cell antigen and cell associated glucosyltransferase enzyme (CA-GTF) of *Streptococcus mutans* in preventing recolonization of *S.mutans* in dental plaque of human volunteers. The experimental period was up to 210 days. At the end of the experimental period, in the experimental group treated with IgY to whole cell *S.mutans* reduction in reestablishment of *S.mutans* from base line value was 67.66% and the group treated with IgY to CA-GTF it was 89.66%. But in the control group treated with non-immune IgY reestablishment was greater than its baseline value with the increment of 9.67%. The promising results in the experimental studies vividly reveal that immune IgY to CA-GTF has significant influence in preventing caries development.

KEYWORDS: IgY, CA-GTF, *Streptococcus mutans* and WC antigen



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INTRODUCTION

Face is the index of mind and oral cavity is the index of general health. Oral health has a significant effect on general health and healthy quality life¹. Dental caries, an important oral disease is a devastating disease of mankind that dates back to antiquity and is still an ongoing significant public health problem of global concern, particularly in developing countries affecting the growth and well being of millions of children^{2, 3}. Alarming increase in dental caries indicates the challenging situation to be faced by the world especially by developing countries. Dental caries, the single most prevalent and costly oral infectious disease⁴ not only causes disturbances of normal functions in the oral cavity, excruciating pain, discomfort, poor nutrition, disruption of sleep, but also causes the major medical complications such as Infective endocarditis, cardio vascular diseases and pneumonia^{5,6,7}. As a consequence, the need to focus more on the prevention of dental caries is now widely accepted as an important step towards the war against dental caries and is of utmost priority deserving the attention of scientific community. It seems unlikely that all the preventive measures in existence put together have been found sufficient to eliminate this multifactorial disease involving diet, host factors and cariogenic bacteria. *S.mutans* is the chief etiological agent of dental caries by virtue of its possession of cariogenic determinants such as adhesins⁸, glucosyltransferase enzymes^{9, 10}, mutacin and glucan binding proteins¹¹. *S.sobrinus* and lactobacillus are less cariogenic and opportunistic. The important cell associated glucosyltransferase enzyme (CA-GTF) of *S.mutans* plays a key role in synthesizing water insoluble glucan from fermentable carbohydrates present in the oral environment. Water Insoluble glucan is the significant constituent of the dental plaque which facilitates further accumulation and aggregation of cariogenic bacteria mediated by glucan binding proteins. Since GTFs from the two cariogenic streptococcal species in humans, *S. mutans* and *S. sobrinus* have very similar sequences in the functional domains in the catalytic region

(CAT), immunization with GTF protein or subunit vaccines from one species can induce a measure of protection for the other species¹². As most of the approaches against dental caries are of treatment in nature which is quiet expensive, the dire need for new alternative and potential preventive approaches for challenging the cariogenic bacteria has been universally accepted and now the focus is on active and passive immunotherapy. Absolute safety should be ensured with respect to immunotherapy as dental caries is not a life threatening one. Newer approaches are focused on the application of passive immunotherapy and successful passive immunization may ride over active immunization which seems to have more risk than benefits. Passive immunotherapy with bovine milk antibodies¹³, monoclonal antibodies¹⁴ and plant antibodies¹⁵ have been found to decrease *S.mutans* count in dental plaque as well as reduction in caries lesions. Although egg yolk antibodies, now called immunoglobulin Y (IgY) was discovered in the late 1800s, alternative possibility of producing antibodies has attracted attention only in the last two decades¹⁶. Egg farming can be switched over as antibody farming for prophylactic and therapeutic applications in human and veterinary medicine, in counterbioterrorism and also for diagnostic purposes¹⁷. Chicken antibody is financially viable with sustainable availability and huge quantity of antibody is obtained from hens, approximately a total potential harvest of 20 g total IgY/year¹⁸ of which 1–10% is expected to be antigen specific¹⁹. Eggs of avian origin may be used as an antibody source and purification procedures such as water dilution methods are simple, cheap and nontoxic. There is apparently no systemic immune response with respect to orally administered antibodies²⁰. Immune complexes containing IgY do not activate the mammalian complement system and do not interact with mammalian Fc and complement receptors that could mediate inflammatory response²¹. The use of IgY for passive immunization has been studied extensively demonstrating its effectiveness in preventing or treating infectious diseases

caused by various pathogens in animals and in humans. In clinical trials in Cystic fibrosis patients, a mouth rinse containing purified anti-*Pseudomonas aeruginosa* IgY given on a continuous basis significantly reduced or prevented *Pseudomonas aeruginosa* colonization, thereby reducing the need for antibiotics^{22, 23, 24}. Specific IgY is effective for immunotherapy for long treatment periods without negative side effect²³. Oral administration of IgY antibodies against rotavirus produced a significant protective effect in children²⁵. An effective local protection against plaque formation related to dental caries was achieved with anti-*S.mutans* IgY²⁶. Anti *S. mutans* IgY spray in adult volunteers produced significant decrease in *S. mutans* colonies in the test group after three weeks of IgY application²⁷. Tooth paste incorporated with anti *S. mutans* IgY was found to be effective in reducing caries in deciduous teeth in human volunteers²⁸. Use of Lozenges containing IgY to CA-GTF of *S. mutans* showed significant decrease in *S. mutans* count in the test group²⁹. Hence egg yolk IgY seems to be well suited for peroral immunotherapy²¹.

MATERIALS AND METHODS

Generation, purification and characterization of Chicken egg yolk antibodies

White leg horn chickens of 21 weeks old were immunized with whole cell antigen and cell associated glucosyltransferase enzyme of standard strain *Streptococcus mutans* serotype c 497 obtained from Microbial Type culture collection and gene bank, Chandigarh, India for the generation of respective antibodies. Booster doses were given whenever there was a reduction in the titer of antibodies. Purity, Protein content, total IgY content, specificity and titer of antibodies purified from egg yolk obtained by both Polyethylene glycol method and water dilution method were determined.^{18,30}. Evaluation of *in vitro* neutralization of virulence properties of *S.mutans*³¹ and *in vivo* efficacy testing of antibodies against experimentally induced caries in rats was carried out³².

Evaluation and comparison of efficacy of Anti Streptococcus mutans IgY and Anti CA-GTF IgY in human volunteers

Ethical clearance was obtained from Sri Ramakrishna Dental College and Hospital ethics committee and written informed consent was obtained from dental college students of age group 20 to 24 who volunteered for the study. They were matched for age, sex, DMFT³³, comparable gingival index³⁴ and plaque index³⁵. 60 volunteers who fulfilled both inclusion and exclusion criteria were included in the study. Examination for dental caries in all the subjects was carried out by a single examiner using a mouth mirror and explorer.

Inclusion criteria

1. Healthy volunteers in whom pre experimental culture of stimulated saliva consistently grew more than $\geq 10^5$ CFU/ml of *S mutans*/ml.

Exclusion criteria

1. Subjects who have taken antibiotics or mouthwash for 5 consecutive days or corticosteroids in the past 30 days
2. Subjects who have a history of sensitivity to any drugs, mouthwash or egg
3. Subjects who have removable prostheses or an orthodontic appliance
4. Subjects who have undergone professional measures to remove plaque and calculus in the past 15 days

DMFS and DMFT index

1. DMF teeth index (DMFT) which measures the prevalence of dental Caries/Teeth.
 2. DMF surfaces index (DMFS) which measures the severity of dental caries. Maximum score: Minimum score = Zero
1. DMFT = 32
 2. DMFS = 48 + 100 = 148

Experimental groups

Participants were randomly allocated into 2 experimental groups and one control group, each group having 20 numbers and the duration of the experiment was 7 months.

Group 1

Control - Mouth rinse incorporated with non-immune IgY. Mouth rinsing - two times a day for 2 minutes for 15 days.

Group 2

Mouth rinse containing IgY antibody against whole cell antigen. Mouth rinsing - two times a day for 2 minutes for 15 days.

Group 3

Mouth rinse containing IgY antibody against CA-GTF. Mouth rinsing-two times a day for 15 days.

Collection of salivary samples

Several studies have shown that number of *S.mutans* in dental plaque does not show any variation in caries better than the number in paraffin stimulated whole saliva³⁶. Hence three samples of stimulated saliva were collected from each subject during a two week period. The baseline *S. mutans* level in each subject was determined from the mean value of the three samples. Participants were asked to refrain from eating for one hour before saliva collection. After chewing with sterile paraffin wax for 3 minutes, 2 ml of induced saliva was collected from the subjects in a calibrated sterile container chilled on ice. The salivary samples of all the patients were identified by a code number during the period of sample collection and processing. The same code number was used for the particular patient

during subsequent saliva sample collection. Samples were transported in ice box within half an hour to the laboratory for immediate processing. To reduce *S.mutans* colony count before starting the experiment, professional mechanical tooth cleaning was done for all the participants followed by mouth rinsing with chlorhexidine gluconate 0.2% mouth rinse (10 ml for 1 minute twice a day in the morning and night for 10 days) after brushing their teeth. They were allowed to spit out, but not to rinse their mouth for 30 min after each application. Study groups rinsed their mouth for 2 minutes with the mouth rinse containing respective IgY immediately after chlorhexidine therapy for 15 days. Composition of the mouth rinse is given in Table 1. The participants were reminded through telephone calls and/or messages to use the mouth rinse. The participants were allowed to continue with their oral hygiene habits and diet during the study period, and to refrain from eating and drinking for 1 hour after rinsing the mouth. Feedback related to compliance and adverse effects, if any, with respect to the mouthwash used was obtained. Paraffin induced saliva was collected and *S.mutans* count was estimated at 4 phases

1. Baseline
2. After PMCT and mouth rinsing with chlorhexidine
3. Immediately after treatment with immune or non immune IgY
- 4) On 41st day, 55th day, 90th day, 120th day, 150th day 180th day and 210th day

Table1
Composition of mouth rinse

Ingredients	Composition of mouth rinse A	Composition of mouth rinse B	Composition of mouth rinse C
IgY to whole cell antigen of <i>S.mutans</i>	-	100mg	-
IgY to CA-GTF of <i>S.mutans</i>	-	-	100mg
Pudinhara	1ml	1ml	1ml
Sterile Glycerine	2ml	2ml	2ml
Sterile Water	1000ml	1000ml	1000ml

Isolation and enumeration of Streptococcus mutans

Saliva was vortex mixed in a cyclomixer for 30 seconds. 100 µl of the vortexed salivary sample was pipetted out using a standard 100 µl pipette and serial dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴

and 10⁻⁵) were prepared in phosphate-buffered saline (PBS). A 100 µl volume from each of the dilutions was pipetted and plated onto Tryptone soya yeast agar incorporated with 20% sucrose, 0.2 units/ml of bacitracin, 10µg/ml of colistin and 30µg/ml of flucanazole. The inclusion of sucrose leads to the formation of

glucans and a distinctive colony appearance that aids in identification. Using a sterile spreader sample was evenly spread employing spread plate technique onto the surface of separate agar plates in duplicates. After 48 hour incubation period in 5% CO₂ enriched atmosphere at 37°C, the agar plates were inspected for the growth of *S. mutans* colonies. The colonies showing distinct cultural characteristics of *S. mutans* were selected and processed for identification. At least five colonies of similar morphology from each plate were examined to confirm the presence of *S. mutans*. Colonies morphologically resembling *S. mutans* were subjected to gram's staining procedure and biochemical tests. Pure cultures of each isolate were identified by their capacity to ferment mannitol, sorbitol, melibiose, raffinose and inulin, arginine hydrolyses, catalase production and negative reaction for hydrogen peroxide production³⁷. Total number of colonies conforming to *S. mutans* per ml of saliva were derived by multiplying number of colonies with dilution factor. Counts were expressed as log₁₀ transformed CFU/ml.

Statistical analysis

The pre-test and post-test data were subjected to statistical analysis for evaluating the difference in salivary *S. mutans* count within the groups and between the groups. The values are the mean + standard deviation of *S. mutans* levels expressed as a percentage of the baseline *S. mutans* level. One way analysis of variance was carried to find out whether there

are any significant differences in the variance of the three groups and if the variances are significant, comparison of the mean values is done to find out which group or groups show an effective reduction in *S. mutans* count following treatment with respective antibody. To find out the best mean/means Post Hoc test and Duncan multiple range test (DMRT) was applied for this purpose.

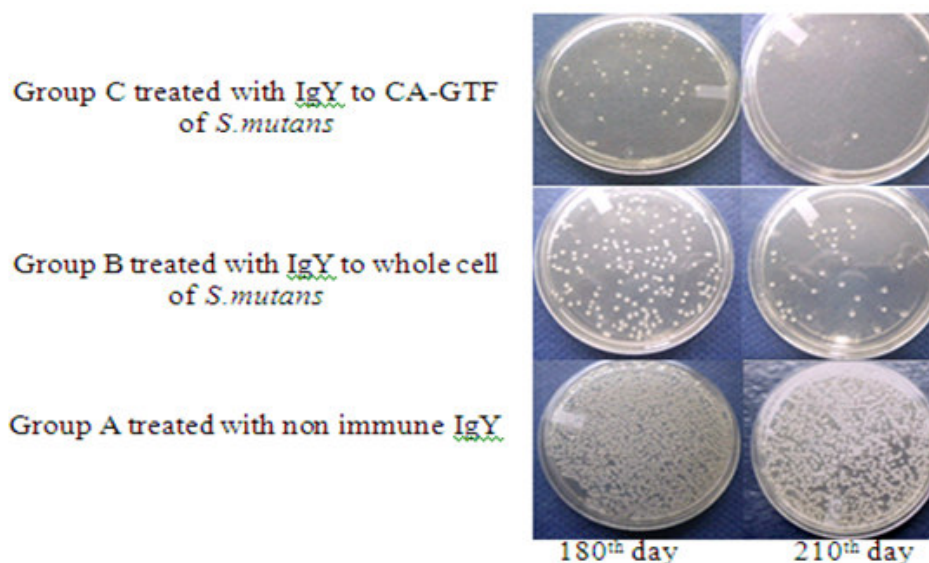
RESULTS

Results of in vitro assays and animal study revealed the higher efficacy of antibodies to CA-GTF against dental caries^{32,33}. Clinical indices of control group and Test groups are given in the Table 2. All the subjects included in the study had *S. mutans* count ranging from 10⁵ to 10⁶. The means of the baseline *S. mutans* count of group A, group B and group C was 8.5x10⁵, 7.7x10⁵ and 7.6x10⁵ respectively and after chemo-mechanical treatment it was 0.045 x10⁵, 0.068 x10⁵ and 0.05 x10⁵. Following chemo-mechanical treatment there was 99.47%, 99.11% and 99.34 % reduction of *S. mutans* count from base line level in the groups A, B and C respectively. The percentage reduction or increment of *S. mutans* was compared between the three groups namely control (Group A) and test groups (Group B, Group C) from the original base line level after treatment with immune or non-immune IgY at regular intervals up to 210th day of experiment (Table 3 and Fig 1, 2).

Table 2
Clinical indices of control and test groups

	Group A- Control	Test Group B	Test Group C
Numbers	20	20	20
Age	23.2 ± 0.42	23.7 ± 0.58	23.1 ± 0.42
Sex F/M	15/5	15/5	14/6
DMFS	15.4 ± 1.46	16.55±3.01	20.1 ± 2.44
DMFT	4.3 ± 0.65	5.7±1.59	6.8 ± 0.76
Gingival index	0.29 ± 0.01	.32±0.01	0.41 ± 0.02
Plaque index	0.52 ± 0.02	0.61±0.03	0.65 ± 0.03

Figure 1
Effect of immune/non-immune IgY in reduction of salivary *S.mutans* level

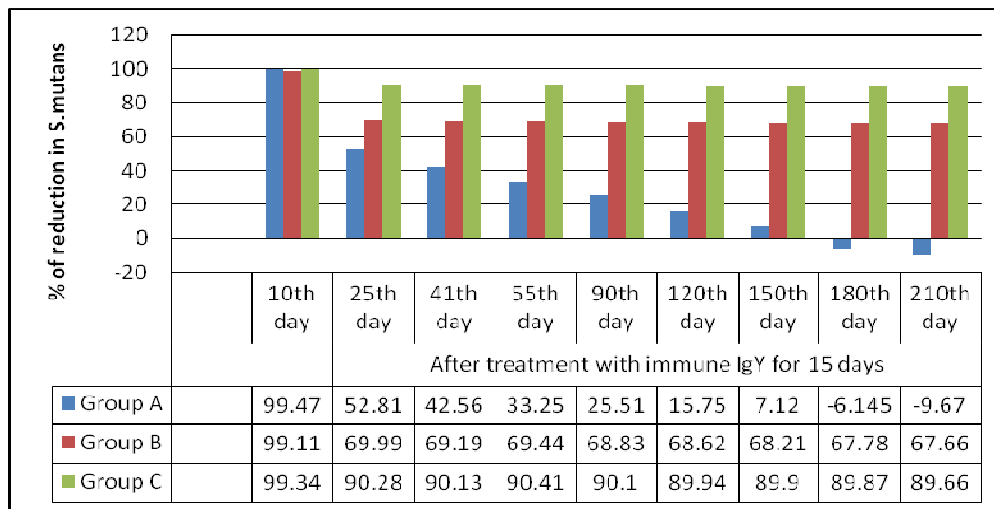


Streptococcus mutans colonies on Tryptone yeast agar showing decrement / increment of Test groups and control group after treating with immune IgY

Table 3
Percent reduction in *S.mutans* count from baseline count following treatment with immune IgY 25th day

Group	N	Mean value	Std deviation	Minimum	Maximum	F statistics	DMRT significance
Group A	20	52.81	10.49	30.4	68.40	167.821	c
Group B	20	69.99	3.88	60.90	80.40		b
Group C	20	90.28	1.35	36.40	92.50		a
41st day							
Group A	20	42.56	11.93	25.9	64.30	211.995	c
Group B	20	69.19	4.07	63.00	78.50		b
Group C	20	90.13	1.39	26.90	92.40		a
55th day							
Group A	20	33.25	9.98	17.70	754.90	410.119	c
Group B	20	69.44	4.14	63.80	80.00		b
Group C	20	90.41	2.32	87.70	98.20		a
90th day							
Group A	20	25.51	9.93	8.8	50	546.807	c
Group B	20	68.83	4.29	63.1	78.5		b
Group C	20	90.10	1.28	87.8	92.5		a
120th day							
Group A	20	15.75	10.78	-5.8	37.10	651.7	c
Group B	20	68.62	4.09	62	77.40		b
Group C	20	89.94	1.17	88.20	91.70		a
150th day							
Group A	20	7.12	13.8	-29	30.7	526.024	c
Group B	20	68.21	4.17	61.3	76.6		b
Group C	20	89.90	1.19	88.1	91.9		a
180th day							
Group A	20	-6.145	14.63	-32.00	29.7	622.809	c
Group B	20	67.78	5.28	53.3	77.7		b
Group C	20	89.87	1.21	88	91.7		a
210th day							
Group A	20	-9.67	12.16	-36.5	6.5	954.99	c
Group B	20	67.66	4.66	56.5	76.2		b
Group C	20	89.66	1.06	88.00	91.3		a

Figure 2
Comparison of the % reduction of *S. mutans* from baseline level after treatment with immune or pre-immune IgY within the three study groups



*In the group A treated with non-immune IgY there was an increase in the count of *S. mutans* after 210 days. But in the group (B) treated with Anti-WC IgY there was a significant reduction of 67.66%. Group treated with anti CA-GTF showed highly significant reduction of 89.66%*

DISCUSSION

Dental caries was on the increasing trend in many countries in South and Central America, Asia, Africa and Middle East³⁸. Several European countries are also facing a rise of dental caries as a reality which is a cause of concern. Dental caries in children, adults and aged of the twenty first century would be enormous, especially those belonging to low socioeconomic status putting them into high caries risk. Globally the reduction in dental caries would help in reducing the heavy budget allotment for dental treatment. There is a dire need for the development and broadening of anti-caries measures and the need is a worldwide one. Regular use of antiseptics is not acceptable as they cause discoloration of teeth, dentures and restorations and cause irritation of taste buds, ulcers in the oral mucosa and also development of drug resistance by bacteria. Immunotherapy is one of the appropriate approaches to combat *Streptococcus mutans*, the prime etiological agent of dental caries. A number of studies have suggested the possibility of using vaccines of the whole cells or other antigens of *Streptococcus mutans* contributing to the pathogenesis of the disease^{39, 40, 41}. Still several challenges are being faced

in the development of effective safe vaccine. Attempts at caries vaccines are still neither successful nor encouraging. There is a need for prevention of dental caries with safe, effective and financially viable method and passive immunotherapy with chicken egg yolk antibody seems to be a good choice and hence in this study egg yolk antibody against whole cell and CA-GTF antigen was generated and tested for its efficacy *in vitro and in vivo*. Water dilution method was used for extraction of IgY for *in vivo* experiments. IgY extraction by water dilution method is simple, inexpensive, suitable for large scale production and safe in the perspective that it does not involve the addition of any chemicals. As antibodies are highly specific, passive immunization with antibodies is a highly attractive and effective alternative approach. It is completely safe particularly for oral immunotherapy as long as the individual is not allergic to egg. It is stable at various pH and temperature and its activity is maintained by the addition of glycerol. It is stable for 6 months at room temperature⁴² and is 2000 fold cheaper than IgG⁴³. Due to its effectiveness, convenience, high yield, high specificity, and possibility of large scale production without causing distress to the animals it is considered as an ideal alternative to other antibodies.

Based on the various studies with antibody to *Streptococcus mutans* which have shown to reduce colonization of *Streptococcus mutans* and caries development in infected rats, monkeys and humans, the present study was carried out to develop a safe, cost effective and efficient passive immunization to prevent dental caries. *Streptococcus mutans* which has specific ability to form virulent dental plaque is the principal cariogenic bacteria for dental caries. The present study was conducted to generate Chicken egg yolk antibodies (IgY) by immunizing chickens with whole cell (WC) and Cell associated GTF (CA-GTF) antigens of *Streptococcus mutans* and to evaluate and compare the efficacy of anti-WC and anti-CA-GTF IgY in decreasing *Streptococcus mutans* in human volunteers. *Streptococcus mutans* serotype *c* was used in the present experiment as they are implicated as the most important causative bacterium in the development of human dental caries^{44, 45}. Synthesis of water insoluble glucan from sucrose by CA-GTF is involved in sucrose dependent adherence and also for binding to the pellicle of tooth surfaces⁴⁶ thus playing an important role for caries development even though initial attachment to saliva mediated pellicle occurs due to surface protein of *S. mutans*. CA-GTF has catalytic and glucan binding domains and antibody to these domains can block the synthetic ability as well as other aspects of protein glucan interactions. It has been shown that antibody against CA-GTF is effective in preventing adherence of *Streptococcus mutans* to tooth surfaces⁴⁷. Administration of specific antibody to the oral cavity interferes with the accumulation and the activity of *S. mutans* and subsequent development of dental caries. Hence the focus was on CA-GTF in addition to whole cell antigen for development of antibodies in egg yolk. In this study reduction of *S. mutans* level from baseline level after chemo-mechanical treatment followed by treatment with pre-immune IgY in group A, immune IgY to whole cell in group B and anti-CA-GTF in group C was determined up to 7 months at specific intervals. There was a significant difference in the mean values of the three groups in all the samples taken at various intervals after

treatment with immune or non-immune IgY. There was a steady increase in recolonization of *S. mutans* and reached the original level at the end of 5th month in the control group after chlorhexidine treatment which correlates with the study⁴⁸ in which a daily dose of chlorhexidine rinse for two weeks has markedly reduced the cariogenic bacteria in the mouth with recolonization in three to six months. In this study human volunteers who used a mouth rinse with immune IgY showed a reduction of *Streptococcus mutans* from baseline level and the % of reduction was 67.66 and 89.66 by the group B and group C respectively whereas 9% increase in *S. mutans* from the baseline was observed in the control group A on 210th day. A short term study showed decrease in the ratio of the percentage of *Streptococcus mutans* per total streptococci in saliva in the short-term (4-hour) test using a mouth rinse containing 10% sucrose and immune IgY⁴⁹. Using tooth paste having IgY to *S. mutans* as ingredient reduced the oral *S. mutans* count significantly⁵⁰. A five-day double-masked placebo-controlled trial in young adults have also demonstrated significant decrease ($P < .001$) in salivary mutans streptococcus scores in participants treated with lozenges containing anti-CA-GTF IgY²⁸ which corroborates well with the present study. This small scale short term study showed preliminary evidence of the efficacy of immune IgY to CA-GTF of *Streptococcus mutans* measured by a surrogate endpoint i.e. inhibition of reestablishment of *Streptococcus mutans* after the existing load was brought down to very low level by chemo-mechanical treatment. A short term topical treatment with antibody resulted in an apparent long term interference with the *Streptococcus mutans* recolonization using monoclonal antibody⁵¹. The best explanation may be the ecological one. Thus antibody blockage of an important event that is heavy accumulation of *Streptococcus mutans* due to CA-GTF or adhesion during the reconstruction of biofilm following immune IgY treatment places indigenous *Streptococcus mutans* at an insurmountable competitive disadvantage for recolonization. The time at which *Streptococcus mutans* is acquired in relation to other common

oral bacteria is critical in its successful colonization and persistence as earlier-arriving species have an ecological advantage over those arriving later^{52,53}. An interesting phenomenon has been observed in young children who do not become naturally infected with *Streptococcus mutans* during the window of infectivity period remain undetectably infected for several years⁵⁴ possibly because its abode in the dental biofilm has been filled by other indigenous flora. As window of infectivity is closed by 30 months of age⁵⁵ preventive strategies that delay the transmission of this bacteria may effectively prevent *Streptococcus mutans* infection throughout childhood. The treatment with chicken egg yolk antibodies to CA-GTF in children during window of infectivity and during secondary dentition may be very effective in preventing the colonization of *Streptococcus mutans* in biofilm and thus dental caries. In adults too it may give long term benefit. But clinical studies have to be conducted in different age groups particularly in children during window of infectivity and for long period in adults for determining its long term effect in prevention of recolonization of *Streptococcus mutans* and dental caries increment. Significant inhibition in reestablishment of indigenous *Streptococcus mutans* in human volunteers by using immune IgY to CA-GTF in the form of mouth rinse is an eye opener for more large scale clinical trials in children and young adults in future to evaluate the efficacy both in terms of reduction of *Streptococcus mutans* count and arresting further development of caries for a prolonged period, so that it can be commercialized. IgY

can also be incorporated in chewing gum, candy, tooth paste and lozenges so that they can be used voluntarily with high compliance. More benefit is expected if passive immunization is used from 12 months to 36 months of age when primary dentition occurs and also during the secondary dentition period when they are prone for colonization with *S. mutans*. The period of 19 to 30 months after birth is described as “window of infectivity”⁵⁶. If colonization is prevented at this stage it will prevent development of caries in primary dentition stage. When permanent teeth erupt, immune IgY may also reduce risks for caries development in later ages⁵⁷. In adults it will arrest further progression of caries.

CONCLUSION

This study has shown the ability of IgY generated against CA-GTF in causing significant reduction in recolonization in dental plaque. More clinical trials are needed using different strength of immune IgY and in different age groups across the population for the successful application of chicken egg yolk antibodies. This would reveal that IgY can effectively combat pathogens that can overcome certain prevailing prevention methods or complement certain methods when there is a need for an effective therapeutic agent.

CONFLICT OF INTEREST

Conflict of interest declared none.

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