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RESEARCH ARTICLE

CREATION AND EVALUATION OF MICROBIAL BIOFILM INDUCED MICROSCOPIC ENAMEL DEMINERALIZATION - AN IN VITRO STUDY

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ABSTRACT

Back ground: Prevention of dental caries requires new researches utilizing experimental set up due to strict ethical issue. **Aim**: In vitro creation of monospecies (Streptococcus mutans) biofilm induced microscopic human enamel demineralization using a batch culture technique and evaluating it using scanning electron micrograph and Energy Dispersive X-ray analysis. **Study design:** Human tooth enamel sections of Group 1 (control group n=nine), Group 2 (Study group n= Nine) were incubated in fresh BHI broth with 5 % sucrose for fifteen days. Test tube with Group 2 is only inoculated with streptococcus mutans every day for fifteen days. SEM-EDAX aided structural analysis for surface morphology and elemental analysis for calcium, phosphorus, oxygen and fluorine content of both control and study groups performed before and at the end of fifteen days. **Result:** A thick, whitish coating of biofilm was visible only on group 2 (Study group), at the end of fifteen days. SEM - EDAX of enamel surface of group 2(Study group) showed loss of superficial structure and decrease in the concentration of calcium, phosphorus ions indicating demineralization unlike group 1 which shows no change in structural and elemental concentration after the incubation period. ANOVA test showed that difference of mean in two groups (1&2) were statistically significant ($p<0.0001$) in calcium, phosphorus, content.

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INTRODUCTION

Prevention of dental caries requires new researches. The complexity of the oral environment, and ethical problems directed the attention to development of experimental caries models, that simulate the human oral microcosm (Gaoyan Tang *et al*., 2003). The experimental models are valuable alternatives to animal model as they can be more efficiently controlled and also because many institutes do not have ethical clearance for performing researches among animals. Therefore, different experimental caries models have been developed (Gaoyan Tang *et al*., 2003).

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complex processes and the factors affecting dental caries. They help us to accurately predict, in a controlled and simplified way, a clinical outcome which can lead us to preventive actions for dental caries (Featherstone, 1996). The development of experimental models should be based on prior knowledge of the oral cavity. As our understanding of the oral cavity progresses, model systems can be improved. Even though a model cannot capture all of the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions. There are a variety of model systems available that can be applied to study dental enamel caries process, each one presenting advantages and disadvantages (Krista *et al*., 2015). Experimental chemical models such as pH cycling and immersion in acid medium are widely used to simulate cariogenic challenges (Featherstone *et al*., 1986). The disadvantage of these models is that they do not simulate the

real demineralization process of the oral environment due to the absence of microorganisms, consequently, concentrating on the physical-chemical aspects of enamel dissolution (Holly *et al*., 1968). This can be overcome by bacterial models in which either planktonic bacteria or microorganisms organized in biofilms can be used. Bacterial models can be used for Investigation of the etiology and prevention of carious lesions, comparison of the cariogenic potential of different bacterial populations and assessment of the cariogenicity of various diets (Katz *et al*., 1986). Studies have shown that microorganisms growing on surfaces as biofilm are generally more resistant to antimicrobial agents than the same cells growing in conventional liquid media (Marsh and Nyvad, 2003). An in vitro model that uses bacterial biofilms is likely to be more representative than chemical or bacterial slurry systems, since dental caries is a bacterial disease and the bacteria which cause it are members of a biofilm community which may lead to altered metabolism compared with free-living microorganisms (Aldsworth *et al*., 2001). Bacterial biofilms are a thick grouping of micro organisms that are very resistant to antibiotics and anti microbial agents and that live on gingival tissues, teeth and restorations, causing caries and periodontal diseases (Katz et al., 1986)). Bacterial biofilm caries models can roughly be divided into two groups: closed batch culture and open continuous culture models. The closed batch biofilm models do offer means of comparing multiple test compounds or conditions simultaneously and require small amounts of reagents and are convenient, reproducible, and economical to use (Blanc *et al*., 2014). There are very few study in India regarding experimental bacterial caries model.

Aim: In vitro creation of monospecies (*Streptococcus mutans)* biofilm induced microscopic human enamel demineralization using a batch culture technique and evaluating it using scanning electron micrograph and Energy Dispersive X-ray analysis.

Objective: To evaluate a laboratory method of creating Artificial dental caries, simulating that in oral environment without compromising the ethical issue which may be used for researches in the context of prevention of Dental caries.

MATERIALS AND METHODS

Preparation of study sample: Thirty caries free, fresh, human premolar teeth, extracted for the purpose of orthodontic treatment were collected as anonymous biomedical waste product of department of maxillo-facial surgery. The enamel surfaces of the tooth samples were analysed using a stereomicroscope (Olympus SZX7; Olympus Corporation, Tokyo, Japan). Non carious tooth without defective cracks, pits, white spots Finally nine non carious teeth were selected. To minimize the risk of transmission of blood-borne pathogens, extracted teeth were disinfected by autoclaving in 121°C/20 psi for 40 minutes following the protocol of The Centre for Disease Control ,USA which neither interfered the enamel hardness (Parsell *et al.,* 1998) nor its demineralization pattern (Moller *et al*., 1998). Each tooth samples were derooted, coronal portion were then cut longitudinally in the mesio-distal plane using diamond discs (KG Sorensen Ind e Com LTDA) mounted in an air-rotor handpiece. Each tooth yielded two sections. All samples were rinsed with distilled water. All together eighteen caries free enamel samples were stored in sterilized , plastic containers with double deionized water until next use.

Creation of central isolated window of enamel: 3mm x 3mm wax pieces were cut from a modeling wax sheet and placed in the middle third of the labial surface of all the forty incisors to form a window. Except on the wax sheet around the crown surface acid resistant nail varnish was coated in 2 layers. Once the paint dried the wax sheet was removed and the samples were ready with a exposed, central isolated window of enamel.

Attachment of wire to tooth sample: The enamel slabs were attached to orthodontic wires with Feviquick, so as to leave the free enamel window to be immersed in the medium without touching the tube walls. The tooth slabs were coded as Group 1 and group 2.

Bacterial Preparation: Freeze dried standard culture samples of *Streptococcus mutans* (MTCC -497) obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh. The optical density of the culture was adjusted to obtain a standard amount of cells of approximately 2.15 x 10^8 CFU/mL

Biofilm Growth and Lesion Production: The enamel slabs with wire assembly (Group 1, 2) were removed from deionized water and immersed in sterile brain-heart infusion broth –BHI (Himedia, Mumbai) with 5% sucrose (Himedia, Mumbai) in two set of test tubes marked A&B .The test tubes were loosely closed with cotton plug to allow gas change with the environment and were sterilized in autoclave at 121°C/20 psi for 40 minutes. Only Test tube B was inoculated with 10 ml of 24 hrs. Incubated culture of *streptococcus mutans* and inoculation was performed at every 24 hours. All tooth slabs had their medium changed at the same time to prevent any kind of contamination and were incubated at 37ºC in an atmosphere of 10% CO2 (Cole Parmer Instruments, USA) in an co_2 incubator. The above steps were repeated 15times for 15 new sets of test tubes for consecutively 15 days for the two groups of tooth sample. Contamination at test recipients was verified at each 24 hours by inoculation in BHI agar media (Merck, Darnstadt, Germany).

Identification of Biofilm: After incubation of 15 days, the tooth sections were taken out and observed for biofilm formation. A thick, uniform, whitish layer was noticed only on enamel Section 2 (non carious experimental group) immersed in test tube marked B (inoculated with bacteria).

Confirmation of inhabitant of Biofilm: The biofilm was scraped and the inoculum was streaked on to the blood agar followed by incubation for 24 hours in a gas pack. Microbial colonies of *streptococcus mutans* was observed along the streak with haemolysis of the RBC in the blood agar. Thus it was proved the biofilm on tooth section marked B comprised of pure culture of *Streptococcus Mutans* without any contamination.

Structural and elemental analysis of tooth sections with SEM & EDAX: At the end of day fifteen and following biofilm confirmation, enamel sections from test tube A&B were removed, separated from wire, dried with hair dryer, coded as group 1&2. The two groups of tooth samples were gold & paladium sputtered in SC 7620 Mini Sputter Coater. Photomicrographs of representative areas were taken at 1500x & 3000 X magnifications with Scanning Electron Microscope (Phenom Pro-X) for structural analysis. Elemental

identification of tooth samples were done with Energy Dispersive X-Ray Analysis (EDAX) (Phenom Pro-X). Although EDAX can assess a wide range of elements, attention were focussed on calcium, Oxygen, phosphorus and fluoride wt%.

Waste disposal: Enamel block that are being discarded at the end of the study were subjected to the containerization and labeling provisions of the Occupational Safety and Health Administration (OSHA) Blood borne Pathogen Standard.

20.0.1 and Graph Pad Prism version 5. p-value ≤ 0.05 was considered for statistically significant.

RESULTS

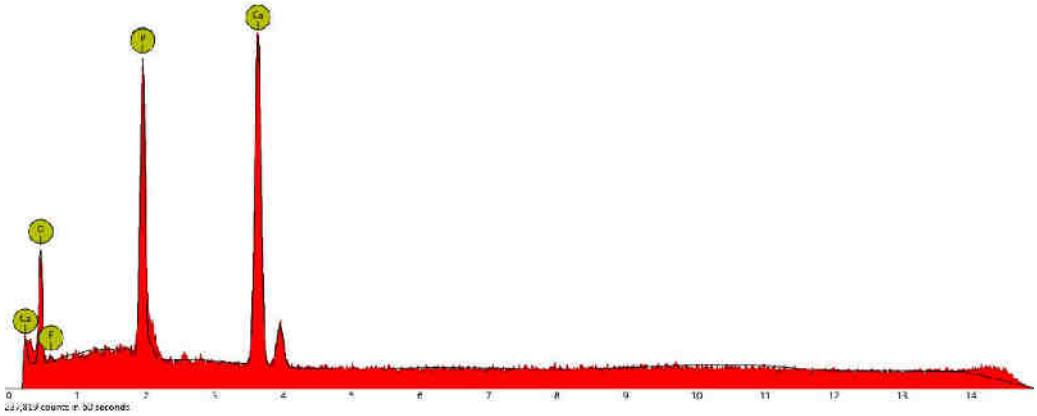
Result of Batch culture technique: After incubation of 15 days a thick, uniform slimy layer was noticed only on tooth Section (Group 2 enamel section) (Fig- 1) immersed in test tube marked B, which was a monospecies biofilm of *Streptococcus Mutans.*

Table 1. Distribution of mean oxygen, Calcium, phosphorus and Fluorine (wt%) in tooth samples 1 & 2 based on result of EDAX

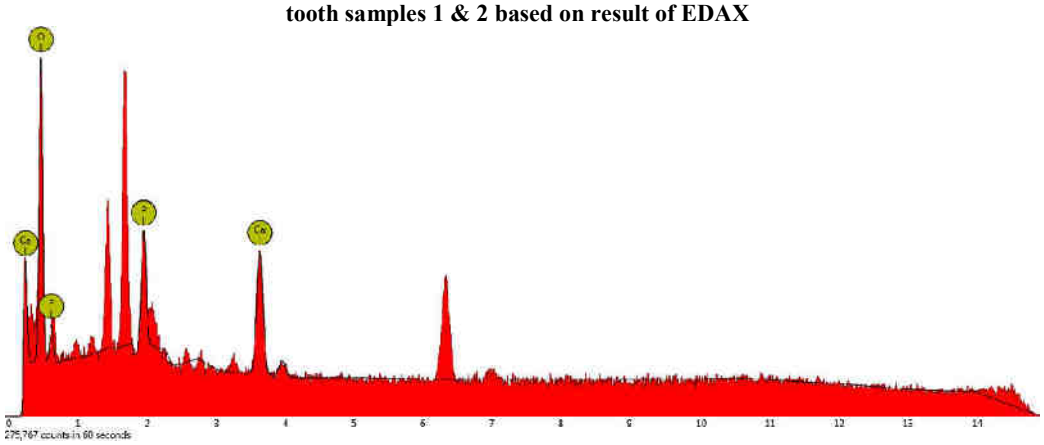
	Group	Number	Mean	Std Dev	Minimum	Maximum	Median	p-value
Oxygen	Group-1	9	40.5667	3.1149	35.3000	45.4000	41.3000	<0.0001
	Group-2	9	63.7778	1.1155	62.1000	65.1000	64.1000	
Calcium	Group-1	9	37.2222	1.9064	34.4000	39.8000	36.8000	<0.0001
	Group-2	9	16.1556	3.3616	12.7000	21.4000	14.7000	
Phosphorus	Group-1	9	19.9667	1.0112	18.4000	21.4000	20.1000	<0.0001
	Group-2	9	12.8667	1.0440	11.3000	14.5000	12.7000	
Fluoride	Group-1	9	1.8000	0.6595	1.0000	2.7000	1.7000	<0.0001
	Group-2	9	6.8889	2.2844	3.2000	8.7000	7.7000	

Table 2. Comparison of Group-1 (control group) vs Group-2 (study group), according to oxygen, Calcium, phosphorus and Fluorine content (wt%) of tooth samples determined by EDAX

	Group-1 Vs Group-2	
	t-test	p-value
Oxygen	21.0461	<0.0001
Calcium	16.3538	<0.0001
Phosphorus	14.6548	<0.0001
Fluorine	6.4207	<0.0001



Graph-I -Showing distribution of mean oxygen, Calcium, phosphorus and Fluorine (wt%) in tooth samples 1 & 2 based on result of EDAX



Graph II. Showing Comparison of Group-1 (control group) vs Group-2 (study group), according to oxygen, Calcium, phosphorus and Fluorine content (wt%) of tooth samples determined by EDAX

a Microsoft excel spread sheet and then analyzed by SPSS

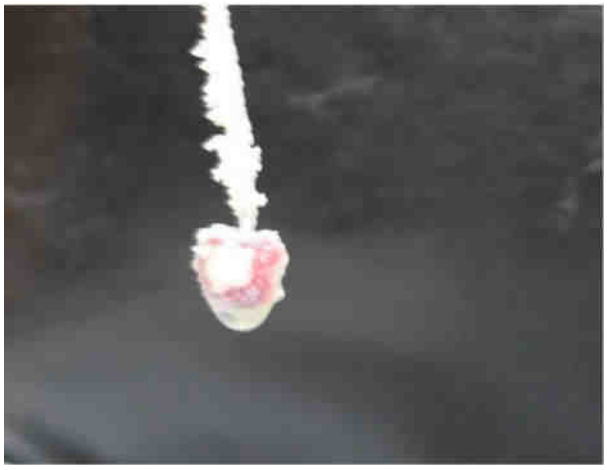


Figure 1. At the end of fifteen days of batch culture technique, visible biofilm formation on enamel section of Group 2 (study group) which was inoculated with bacteria

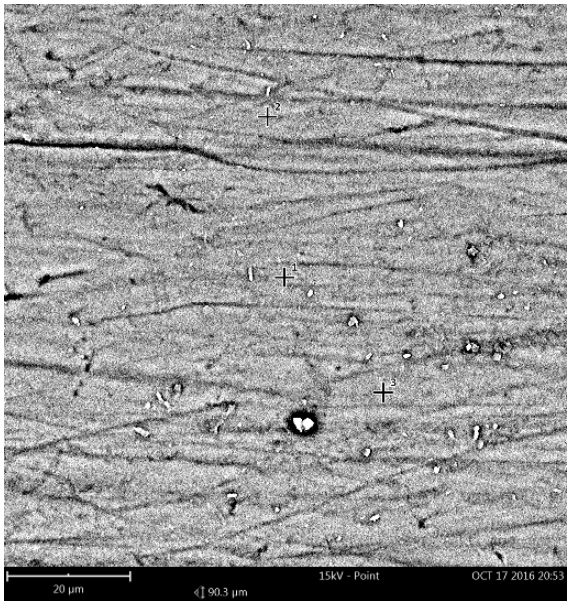


Figure 2.SEM micrograph (3000x) of the sound enamel surface of Group 1.(control group) indicating 'no alterations'

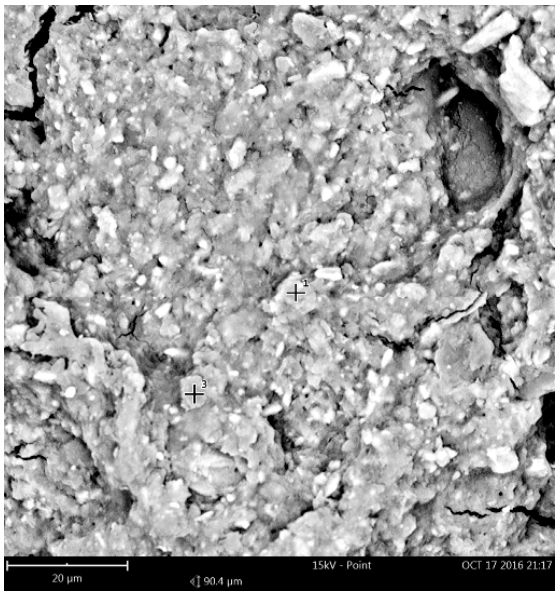


Figure 3. SEM micrograph (3000x) of the enamel surface of Group 2 (study group) indicating moderate increase in roughness of enamel surface

Result of SEM micrograph: SEM micrograph of sound enamel surface of Group 1, Figure -2 shows smooth and sound surface with circumferentially horizontal lines on the surface known as perikymata. Surface topography of Gr. 2 (Figure -3). Shows surface alterations (rough and uneven) with dissolution of both the interprismatic and intraprismatic substance could detected on the surface of enamel resulting marked increase of porosity and concavity.

Result of EDAX analysis: To further evaluate the differences between the control and study groups, the EDAX analysis (energy dispersive X-rays analysis) was done in which the mineral content like calcium, phosphorus, fluoride, and oxygen in weight % of the enamel samples were quantified (Table 1). ANOVA test showed that difference of mean in two groups (1&2) were statistically significant ($p<0.0001$) in calcium, phosphorus, fluoride and oxygen content (Table 2). In Oxygen, calcium, phosphorus and fluoride content, Student's t-test applied to the mean values showed significant differences between Group-1 (Graph-1) versus Group-2 (Graph -2) ($p<0.0001$) with decrease in concentration of calcium, phosphorus and increase in concentration of Oxygen & fluorine content in study group ie Group-2 (Graph -2) .

DISCUSSION

Oral cavity is enriched with numerous microbes, some of which especially acid producing bacteria like *streptococcus mutans* colonize the dental surface within biofilm and in presence of frequently consumed fermentable carbohydrate (sucrose), they locally produce small amounts of organic acid which reduce the Ph in the bacterium's micro-environment. Since *S. mutans* is able to directly adhere to the tooth's hydroxyl apatite matrix, the pH of the tooth surface may be easily reduced to below the critical pH of HA demineralization (pH 5.5) and demineralization begins at the atomic level on the crystal surface inside the enamel and can continue unless halted with the end point being cavitation or dental caries. In vivo caries studies have the advantage of including host factors involved in the natural caries process but have some fundamental limitations. The oral environment is difficult to control and varies greatly with intraoral location over time and between different persons (Carolina Steiner-Oliveira *et al*., 2007). Laboratory models of this process are potentially valuable in understanding the mechanisms involved, in developing and testing procedures to combat and prevent caries (Guggenheim *et al.,* 2004). A useful in vitro model should have the following characteristics: ease of sterilization of the different components, ability to manipulate model components under sterile conditions, ease of access to test specimens, reproducibility of experiments and optimal simulation of the oral environment. Bacterial systems where the mixed natural microbiota are controlled by in vitro environment and nutrient conditions provide a means for studying complex microbial ecosystems such as dental biofilm and its effect on the development of dental caries. Demineralization is characterized by removal of minerals especially calcium salts from dental enamel, in the form of mineral ions. The present in vitro microbiological model using batch culture technique, produced single species biofilms of *S. mutans,* which in turn successfully developed artificial demineralization on human enamel slab as evidenced by SEM attached EDAX analysis. The SEM images revealed increase in pore volume and roughness of enamel structure in group 2 (study group) (Fig. 2). When compared to the group 1. (control

group) (Fig. 1). The EDAX analysis (energy dispersive X-rays analysis) revealed there is a marked reduction in calcium and phosphorus content & marked increase of oxygen content of group 2 (study group) (Graph- 2). Just like the present study, Carolina Steiner-Oliveira *et al*. in 2007 (Carolina Steiner-Oliveira *et al.,* 2007) also developed an in vitro viable microbiological model to produce biofilms to be used in dental researches. Single and multi-species biofilms of S. mutans,S. sobrinus, S. mitis, S. salivarius, S. cricetus and S. sanguinis were grown on bovine enamel slabs during 10 days, in a sterile brain-heart infusion broth, containing 5% sucrose and incubated at 37ºC in an atmosphere of 10% CO_2. This model created demineralization on enamel and the medium can be changed at every 24 hours utilizing either S. mutans or S. sobrinus. In the present study simple monobacterial biofilm models have been developed using Streptococcus mutans. Defined-species biofilm consortia, although simpler than in vivo, have the advantage of allowing detailed control and study of the properties of the individual bacterial species present (Marsh, 1995). In contrast to present study Guggenheim B *et al.* in 2004 utilized Zürich biofilm model which used six microbial species (*Streptococcus oralis*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum*, and *Candida albicans*) to create artificial demineralization.

Present study evaluated biofilm induced microscopic human enamel demineralization using SEM which is a structural and semi-quantitative analysis.A quantitative and elemental analysis using Energy Dispersive X-Ray Analysis (EDAX) is also used in the present study to measure the amount of mineral loss specially that of calcium, phosphorusion which is the characteristic of demineralization. Where as Guggenheim *et al.* (2004) used fluorescently labeled antibodies and confocal laser scanning microscopy (CLSM) to quantify demineralization. The advantages of the present in vitro, monobacterial, biofilm induced microscopic human enamel demineralization - minimum requirement of reagents, and the possibility of controlling contamination, by monitoring the medium at each 24 hours by inoculation in BHI agar media. The present in vitro model could be further improved by including artificial saliva instead of culture media, by including alternate period of remineralization and demineralization using Ph cycle and also by including multiple cariogenic bacteria thereby creating a multispecies biofilm induced demineralization simulating natural demineralization of enamel in oral cavity. In conclusion It can be revealed that the present in vitro model could produce biofilm induced microscopic human enamel demineralization. The present lab model could be used to study the effect of different preventive protocol on microbial demineralization of enamel.

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