

In vitro model for pit and fissure caries

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Abstract

This paper describes the preliminary steps toward developing an in vitro model for producing pit and fissure caries. Four groups of 12 intact molars mounted in acrylic and pit and fissure areas were painted with Streptococcus mutans-inoculated culture medium. In Group A, the bacterial inoculum was covered by agar. In Group B, carboxymethyl cellulose was used instead of agar. In Group C, agar was used and covered by a filter paper disc and collodion. Group D was the same as C except carboxymethyl cellulose was used instead of agar. Groups A and B were incubated at 37°C. Groups C and D were placed in artificial saliva and incubated in a shaking bath. The study was conducted for 8 weeks and the media were changed twice a week. Group C had the largest number of carious lesions (both microscopic and clinically). However, the difference from the other groups was not significant ($P > 0.05$).

The multifactorial nature of the dental carious process poses a great challenge to the researcher. Isolating and measuring the effects of the primary and secondary etiologic factors associated with caries is difficult.

Dahl and Silverstone (1979) developed a method for pit and fissure caries in sound teeth by using acidified gels. Their histopathologic study of the carious area showed that the progression of lesions occurred in vertical depth and not in width (wall areas). Thus the cavities they observed were not similar to natural caries, wherein the cavity progression occurs in all directions. The development of a method to produce occlusal cavitation similar to that observed in clinical caries is, therefore, an urgent need. If such a procedure could be developed and standardized, the effects of proposed preventive agents applied to occlusal surfaces could be assessed by comparing cavity formation on these surfaces with those in control specimens tested with placebo agents.

It was the overall goal of this study to approach

the development of a method for producing occlusal cavities in sound, extracted human teeth.

The specific objective of this study was to develop a method for producing in vitro occlusal caries with the following characteristics:

- A. The lesions should have the same characteristics as naturally occurring caries, both microscopically and clinically.
- B. The initiation of caries should occur as quickly as possible.

Methods and Materials

Forty-eight sound, extracted human first, second, and third molars from a larger population of teeth were obtained from the Department of Oral Surgery of the Indiana University School of Dentistry. The teeth had been extracted either due to periodontal or prosthodontic problems. They were kept in a 3% aqueous formaldehyde solution and stored in sterilized distilled water until use. The teeth were examined by 2 independent examiners, both clinically (visual and tactile) and microscopically. Visual-tactile examination was done with a sharp explorer #MG2 with the aid of an ordinary bright light. Microscopic evaluation was done using a binocular dissection microscope at 15× magnification. The selection by visual-tactile methods of teeth which were free of surface decalcification or cavitation was based on the criteria for determining pit and fissure carious lesions established in 1968 by the American Dental Association's Council on Dental Research and Council on Dental Therapeutics (1968). Teeth which did not meet any of the criteria for caries were regarded as being caries-free.

By sample selection the teeth were distributed into 4 groups, 12 to a group. The strata were third, second, and first molars. The roots were removed with

a separating disc and each tooth was mounted on an individual acrylic base. A plastic cast was constructed to keep the bases always in the same position. In addition, the teeth were mounted so that the center of the occlusal surface was at the same distance from the base. In this way, approximately the same focal distance could be used for all of the specimens during microscopic evaluation at the different periods of evaluation. The teeth then were sterilized with ethylene oxide.

Cariogenic Challenge

The occlusal pits and fissures of each tooth were covered with a layer of artificial plaque formed by *Streptococcus mutans* and a solid or semi-solid medium containing sucrose. To prepare "artificial plaques" a stock culture of *S. mutans* 6715, grown for 18 hr in a complex medium (Jordan's)^a containing 0.25% glucose, was carefully painted with a sterilized camel hair brush over the pit and fissure area. A layer of Jordan's medium, to which 2.0% agar^b and 20.0% glycerine was added as a humectant was then applied over the initial inoculum. In some groups, 2.0% carboxymethyl cellulose (CMC)^c was added to Jordan's medium instead of agar. The 48 teeth were divided randomly into 4 groups as follows:

Group A. The liquid medium, consisting of an 18-hr culture of *S. mutans* in 0.25% glucose-containing Jordan's (Jordan et al. 1960) medium was carefully painted on the occlusal pit and fissure area with a sterile camel hair brush. Subsequently, the agar medium was melted and tempered to 55°C. The agar then was inoculated with 1 ml of 18-hr culture of *S. mutans* and applied to the pit and fissure area with a sterile camel hair brush. Once the agar solidified, the specimens were placed in a presterilized 600-ml beaker over a layer of wet cotton to keep the environment

^a Jordan's medium formula:

K ₂ H PO ₄	5.0 g
Yeast extract	5.0 g
Trypticase	5.0 g
Sucrose	5.0 g
Jordan's salt mixture 0.5 ml adjust to pH of 7.2 with 0.1 N ClH	
q.s. to 1000 ml	
Jordan's salt mixture:	
MgSO ₄ ·7H ₂ O	0.800 g
FeCl ₃ ·7H ₂ O	0.040 g
MnCl ₂ ·4H ₂ O	0.019 g
q.s. to 100 ml	

^b Jordan's medium with agar:

Same as Jordan's medium plus:	
Agar	20.0 g
Sucrose	50.0 g
Glycerine	20.0 g
Bromocresol purple	0.36 g

^c Jordan's medium with carboxymethyl cellulose:

Same as Jordan's medium plus:	
CMC	20.0 g
Sucrose	50.0 g
Bromocresol	0.36 g
(Type 7MF, Hercules Corporation)	

TABLE 1. Mean Number of Affected Tooth Surfaces and Cavities Observed in Tooth Surface Microscopically After 7 and 8 Weeks Exposure to the Cariogenic Challenge

Group	Total Number of Affected Surfaces		Mean Number of Cavities Per Tooth	
	Week 7	Week 8	Week 7	Week 8
A	8	8	0.91 ± 0.25*	1.16 ± 0.40
B	7	7	0.75 0.21	0.75 0.21
C	11	11	1.25 0.21	1.58 0.28
D	9	10	0.91 ± 0.19	1.08 ± 0.19

* Standard error of the mean (12 specimens were used per group). Values within brackets are not significantly different at $P > 0.05$ (chi-square test, 1-way analysis of variance, or Welch test as it may correspond).

moist. The beaker was sealed and incubated at 37°C for 2 days, at which time the specimens were removed from the beaker and the teeth were brushed with a standard multi-tufted, medium-hardness, synthetic toothbrush and sterile deionized water. The empty beakers were autoclaved and the procedure repeated for another 2 days. This was repeated for 8 weeks. Evaluations were made each time the medium was changed. Any changes, both microscopic and clinical, were recorded. In addition, 2 examiners at weekly intervals observed the progression of the microscopic and clinical lesions that developed.

Group B. This group was treated in the same manner as Group A except that carboxymethyl-cellulose (CMC) was used instead of agar.

Group C. The specimens again were treated in the same manner as Group A except that the layer of agar was covered by a disc of sterile filter paper over which a thin layer of collodion was painted. Then the specimens were placed into a presterilized 600-ml beaker containing 300 ml of sterilized artificial saliva.^d Finally, the beaker was sealed with aluminum foil and adhesive tape and incubated at 37°C in a thermostatically controlled shaking bath at a speed of 40 strokes/min.

Group D. The procedure was the same as in

^d Preparation of "Artificial Saliva"

- a. Stock solutions
 - i. CaSO₄·2H₂O 9.0 g diluted to 4.5 l with deionized water (DIH₂O)
 - ii. NaCl 11.25 g diluted to 1 l with deionized water (DIH₂O)
 - iii. KCl 4.54 g diluted to 1 l with deionized water (DIH₂O)
 - iv. KH₂PO₄ (Mon) 9.315 g diluted to 1 l with deionized water (DIH₂O)
 - v. Na₂HPO₄ (Di) 11.007 g diluted to 1 l with deionized water (DIH₂O)
- b. Procedure to make 1.8 l of "artificial saliva"
 - i. Place 1715 ml of deionized water in a 2000 ml beaker
 - ii. Add 45 ml of CaSO₄·2H₂O stock solution
 - 10 ml of NaCl stock solution
 - 10 ml of KCl stock solution
 - 10 ml of KH₂PO₄ stock solution
 - 10 ml of Na₂HPO₄ stock solution

TABLE 2. Mean Number of Affected Tooth Surfaces and Cavities Observed in Tooth Surface Clinically After 7 and 8 Weeks Exposure to the Cariogenic Challenge

Group	Total Number of Affected Surfaces		Mean Number of Cavities Per Tooth	
	Week 7	Week 8	Week 7	Week 8
A	6	7	0.58 ± 0.19*	0.66 ± 0.18
B	5	5	0.41 0.14	0.41 0.14
C	8	10	0.66 0.14	0.83 0.11
D	5	7	0.50 ± 0.19	0.75 ± 0.21

* Standard error of the mean (12 specimens were used per group). Values within brackets are not significantly different at $P > 0.05$ (chi-square test, 1-way analysis of variance, or Welch test as it may correspond).

Group C except that the carboxymethyl cellulose-containing medium was used.

The study was conducted for 8 weeks, after which the final evaluation was made.

Evaluation

The criteria for dental caries evaluation were mentioned at the beginning of this section. The evaluation of both microscopic and clinical caries was made in terms of number of teeth with occlusal caries per group and mean number of independent cavities per tooth.

Criteria for Microscopic Dental Caries¹. 1. Enamel surface alteration in terms of opacity, which is a manifestation of enamel demineralization. 2. Discontinuity of the occlusal enamel surface caused by a loss of tooth substance detectable when viewed at 15 magnifications. 3. Deepening and widening of pits and fissures. 4. Cavitation in which both enamel and dentin are affected.

Statistical Analysis. The data were analyzed by a 1-way analysis of variance preceded by a Bartlett chi-square test to determine that the variances were homogeneous. If this was not the case, the Welch test was used instead of the analysis of variance. The significance of intermean group differences was performed to determine whether the differences between the number of occlusal surfaces with and without cavities in the different groups were significant.

Results

Table 1 presents the total number of microscopically detected carious occlusal surfaces per group, and the mean number of microcavities per tooth at the seventh and eighth week of evaluation. The total number of affected surfaces ranged from 7 to 11, with none of the differences being significant at the 0.05 level of confidence. The mean number of cavities per

tooth ranged from 0.75 to 1.58. Again, none of the differences were statistically significant ($P > 0.05$).

Table 2 presents the total number of clinically detected carious occlusal surfaces per group and the mean number of cavities per tooth after 7 and 8 weeks of testing. The number of affected occlusal surfaces ranged from 5 to 8 at the 7-week and 5 to 10 at the 8-week evaluation periods. The mean number of cavities per tooth ranged from 0.41 to 0.66 after 7 weeks of testing, and 0.41 to 0.83 after 8 weeks. The intergroup differences at either evaluation time were not statistically significant in either category ($P > 0.05$).

Discussion

Two types of conditions were tested and compared: a stationary model in which the specimens were incubated in a moist environment, and a dynamic model in which artificial saliva covered the specimens and the incubation was conducted in a shaking bath (which was used to accelerate the diffusion of acid into the plaque and dissolved material out of plaque). The cariogenic challenge was provided in the form of an artificial plaque placed over the occlusal surfaces (Wilson 1970). In one case, this plaque consisted of a layer of agar to which glycerine was added as a humectant. It was hoped that the addition of glycerine would prevent drying of the artificial plaque. Drying would cause the plaque to separate from the tooth.

In the second case, carboxymethyl cellulose was used for the same purpose. *S. mutans* 6715 was used as the bacterial inhabitant of the plaque in both cases. In general terms, the cariogenic challenge performed as expected and localized lesions occurred in pit and fissure areas. It is believed that this situation, wherein plaque and its by-products are responsible for producing caries, reflects more closely the formation of natural caries than a system in which only acids are used to obtain that effect (Wefel et al. 1979).

The results of this study showed that the group using agar and the shaking bath had more affected surfaces and more cavities per tooth than the groups not using the shaking bath. The groups using agar had more lesions than those using carboxymethyl cellulose (CMC). However, these differences were not statistically significant. All combinations produced cavities, although all groups—especially Group C (agar and shaking bath)—produced some surface decalcification. This finding is in agreement with other studies in which attempts to produce caries in vitro were made. Sidaway et al. (1964) mentioned that the altered enamel lying beneath an in vitro bacterial plaque looked decalcified, chalky, and closely resembled that seen in natural caries. He also noted that in more advanced experimental lesions, the surface of the enamel was opaque and the surface layer was soft.

¹ Pigman et al. 1952; Gray and Francis 1963; Wilson 1970.

Most of the recent studies have concentrated on producing in vitro carious lesions by the use of an acidified gel.² They claim that formation of subsurface decalcification is similar to what occurs in the oral environment. However, none of these studies have completely simulated the natural oral condition. The lactic acid used in these studies is only one of the acids produced by plaque bacteria. The formation of typical cavitation at localized points has not been achieved.

Many investigators have concurred that dental decay occurs at a faster rate in the artificial mouth than would normally be expected in the human mouth. This is attributed to the lack of features such as the lips, tongue or buccal mucosa. In addition, a medium is used to support bacterial life rather than saliva with its enzymes and antibodies. To what extent this faster rate of decay modifies the results is not known. It should be noted that there is a definite variation in caries response of different teeth within individual mouths, even when subjected to identical treatments. This variation is probably due to a number of factors including biological variation of the teeth and different tooth environments. In the present study, a number of teeth in the same group (mouth) did not develop caries.

The experimental model used in this study proved to be uncomplicated and was simple to set up, maintain, and standardize. It also involved a low operational cost. The model's main disadvantage was the generalized surface decalcification which occurred in Groups C and D. Decalcification was especially severe in Group D where the carboxymethyl cellulose was used as a humectant. The generalized surface decalcification is believed to have been caused by the low pH (< 4.8) of the artificial saliva in which the specimens were incubated for 3 days, when there was no continuous flow of saliva in the model.

Summary and Conclusion

Four groups of 12 intact first, second, and third molars, previously extracted for periodontal or prosthetic reasons, were mounted in acrylic bases with a plastic cast separating the bases. The teeth were exposed to a cariogenic challenge consisting of a layer of artificial plaque composed of *S. mutans* and a solid medium. The medium contained 2% agar plus 20% glycerine added to 5% sucrose-containing Jordan's medium. A semi-solid medium also was prepared by adding 2% carboxymethyl cellulose to the original medium. The 4 groups tested were:

Group A. The inoculated liquid medium was painted over the pits and fissures with a sterile camel

hair brush. Then a thin layer of melted agar medium was applied over the liquid medium. The specimens were incubated at 37°C for 2 days.

Group B. These teeth were prepared in the same way as Group A except that carboxymethyl cellulose was used instead of agar to provide a semi-solid consistency to the medium.

Group C. This group also was prepared like Group A except that the layer of agar was covered by a disc of sterile filter paper over which a layer of collodion was painted. The specimens were placed in a presterilized 600-ml beaker containing 300 ml of sterile artificial saliva.

Group D. These teeth were prepared in the same way as Group C, except that carboxymethyl cellulose was used instead of agar.

After 8 weeks Group C had the highest number of lesions both microscopically and clinically. However, the differences with the other groups were not significant ($P > 0.05$).

This study constituted a first step toward the development of an in vitro model for producing pit and fissure caries. Additional work is needed to improve the model and alleviate problems which were encountered.

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² Silverstone 1966, 1967, 1968.

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