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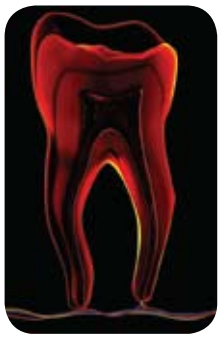

Microleakage of Human Saliva in Coronally Unsealed Obturated Root Canals in Anaerobic Conditions

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ABSTRACT The purpose of this study was to determine the time required for anaerobic bacteria in natural human saliva to contaminate root-filled teeth. Thirty-two single-rooted teeth were cleaned, shaped, filled, and exposed to human saliva for 120 days. Teeth that had not leaked were subjected to polymerise chain reaction examination. Sixty-six percent of the experimental group were totally contaminated. A PCR examination revealed there was no contamination in the apical 3 mm of leakage-free teeth.

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Coronal leakage of saliva has been suggested as being a significant factor in the emergence and persistence of apical disease associated with root-filled teeth.[1] That is, if the coronal portion of the root canal system is exposed to the oral flora, ingress of bacteria can occur with subsequent exposure of the periradicular tissues to their metabolic byproducts.[2] Contamination can occur between the time of obturation and the placement of a definitive coronal restoration.[3]

Recent studies drawing upon advanced microbiological techniques for anaerobic species have revealed that the

composition of root canal microbiota after failed treatment differs from that normally found in untreated teeth and that the polymicrobial flora are predominantly anaerobic and oxygen sensitive.[4-8]

The aim of this experiment was to determine the length of time required for anaerobic bacteria present in natural human saliva to penetrate through the entire root canal system in an ex vivo model.

A variety of materials such as ink and methylene blue have also been used to study microleakage in alloys, resins, root canal filling materials and temporary filling substances.[9-13]

Although the previously mentioned materials may be good tools for comparing relative leakage, they cannot give a true picture of the leakage, which occurs clinically.[14]

Consequently, bacterial or saliva leakage studies could be more meaningful and clinically more relevant.

Most of the articles assessing coronal microleakage by bacteria however, have made use of certain species of bacteria that are not known as being the root canal flora, nor are these bacteria aerobic.[15-17] Furthermore, saliva leakage studies have not been carried out in anaerobic conditions.[2,18]

Materials and Methods

Preparation of the Teeth

Thirty-two extracted human maxillary and mandibular incisors and cuspids with fully formed apices, straight and single-root canals were used in this study. The teeth were collected over a 210-day period, extracted for periodontal or prosthodontic reasons.

The teeth were kept moist by storage in 0.9 percent NaCl solution, throughout the study. The teeth were divided into one experimental group of 22 teeth and two control groups each of five teeth. The teeth were immersed in 2.6 percent sodium hypochlorite solution (NaOCl) for approximately 30 minutes in order to remove organic material from their root surfaces.

Any remaining soft tissue was carefully scraped with a curette to avoid the removal of the cementum from the last 3 mm of the root. The crowns of the teeth were removed and the coronal surface of each root was made perpendicular to the long axis of the root with a high-speed handpiece and a multipurpose bur using air and water spray. Coronal access was made using a No. 245 bur (Dentsply Maillefer, Tulsa, Okla.) in a high-speed handpiece under copious water spray.

Working lengths (WLs) were calculated by subtracting 1 mm from the length at which a size 20 K-File (Dentsply Maillefer, Ballaigues, Switzerland) just exited the apical foramen. The canals were instrumented to size 40 K-File so that a standardized diameter of the apical end of the canals could be obtained. Coronal flaring was accomplished with Gates-Glidden burs, sizes No. 2 and 3. A total of 10 ml of 2.6 percent NaOCl solution was used for irrigation between each file size.

The root canals of the 22 teeth in the experimental group and the five teeth in the negative control group were dried with paper points and the canal walls coated with AH 26 sealer (Dentsply De Trey, Konstanz, Germany). Master gutta percha cones were fitted to 0.5 mm from the working length, nickel-titanium finger spreaders (Dentsply Maillefer, Switzerland) were placed within 1 mm of the working length with the master cone in place, and the canals were obturated using lateral compaction of gutta percha (Coltène/Whaledent, Langenau, Germany).

Accessory cones were added until the spreader could not penetrate more than 1 mm of the working length. Excess coronal gutta percha was removed with a heat carrier before it was vertically condensed using a hand-plugger (M-series, Dentsply/Maillefer, Switzerland).

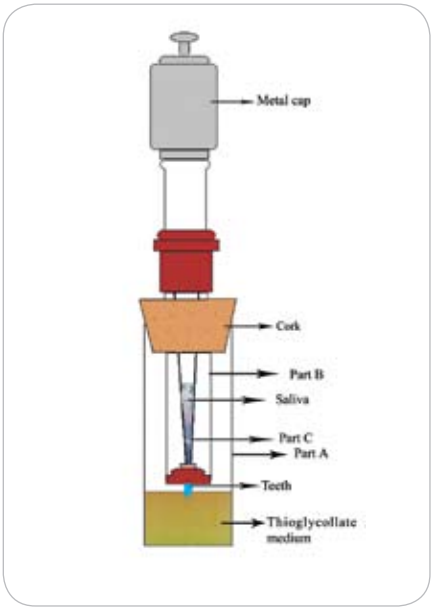

FIGURE 1. The leakage apparatus consisted of three main parts. Part A holds the sterile thioglycollate medium; part B supports the specimens, and part C delivers the fresh human saliva directly to the orifice of the root canal.

The root surface was wiped with gauze and isopropyl alcohol to remove excess sealer. The length of all samples were made equivalent to 10 mm using a high-speed handpiece and a multipurpose bur.

The teeth were wrapped in gauze dampened with sterile saline, enclosed in sealed tubes and placed in an incubator at 37°C for 48 hours to allow the sealer to set.

All the roots were instrumented and obturated by one dentist, with an endodontist supervising all procedures. Five roots, obturated with single gutta percha cones without any root canal sealer, served as the positive control group. The orifices of the negative control teeth were sealed completely with sticky wax (Razi Chemical Co., Tehran, Iran); the other teeth did not receive an interim restorative material.

The external surfaces of the roots were coated with two layers of nail varnish except for the coronal access cavities and the apical 3 mm.

Preparation of Specimens

The apparatus used was a variation based on that used by Gilbert et al. to which several modifications were applied.[17] This model consisted of three main parts (FIGURE 1). Part A was used to hold the sterile thioglycollate medium; part B was used to support the specimens, and part C was used to deliver the fresh human saliva directly to the orifice of the root canal.

First, the teeth were placed in a hole prepared in the plastic head of penicillin vials (Jaber Bin Hayyan Pharmacy, Tehran, Iran) and fixed with chemically active composite resin (Panavia F, Kuray Medical Inc., Kurashiki, Japan). Then, the plastic heads were fixed to the end of part B with the same material. Sticky wax was utilized after Panavia F to seal the sections. The inner section, part C, which fit into the lumen of part B, was advanced to sit directly on the orifice of the tooth. After the construction of the delivery system, the sterile thioglycollate medium was placed into part A to a level of 2 to 3 mm above the apical foramen of each filled root canal.

Fresh human saliva collected every other day from a healthy employee of the Faculty of Dentistry, was carefully placed into the top portion of part C. Every four apparatuses were kept in a jar and then placed in the incubator. Anaerobic conditions in the jars were maintained by the Anoxomat system (Mart Microbiology BV, Lichtenvoorde, Netherlands). The saliva was then replenished every two days under the laminar flow hood.

The opacity of the broth in part A was checked twice a day for 120 days and any changes were recorded as an indicator of entire root canal recontamination. Teeth that had not leaked at the end of the experiment were subject to polymerase chain reaction, PCR, examination to discover the point to which they were contaminated.

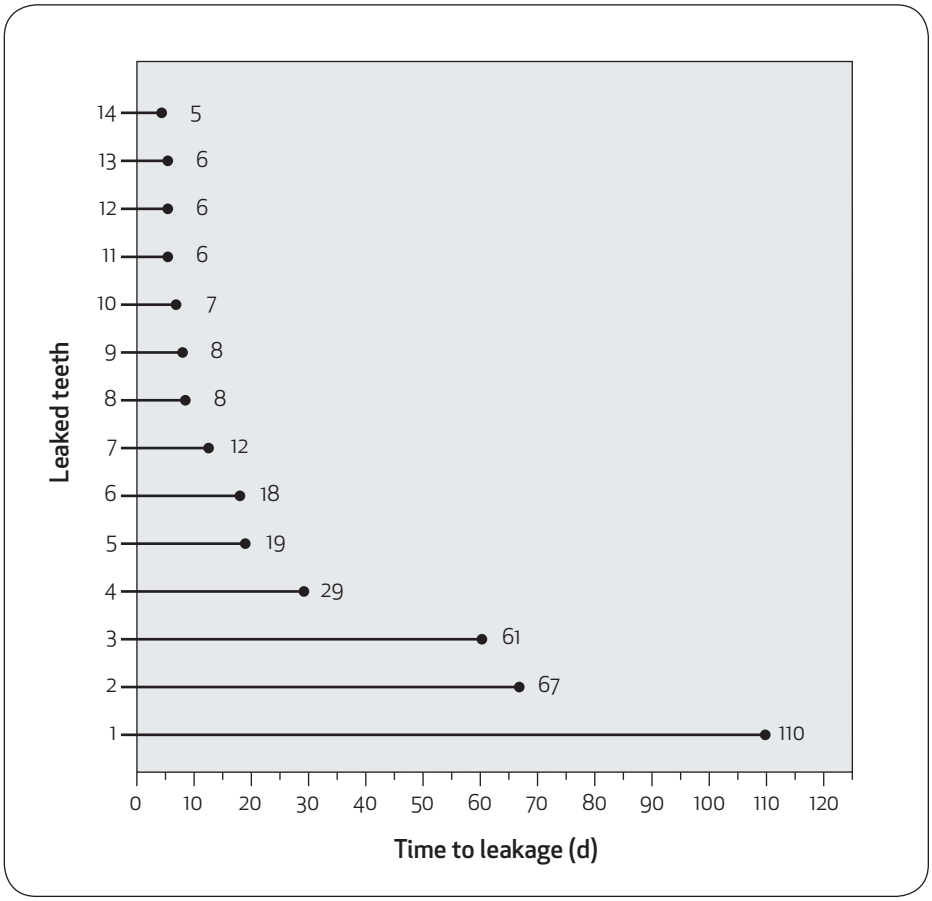

FIGURE 2. The time of leakage in leaked teeth.

Before PCR was performed, the last 3 mm of the aforementioned teeth was cut with a sterile DFS disk bur (TUV, Germany) so that the gutta percha could be extracted using a size 20 K-file.

The broth of the negative control teeth and from the teeth in the experimental group that had not leaked, was removed from part A and mixed with several drops of saliva from part C so that the capability of the system could be confirmed in a separate evaluation.

DNA Extraction and PCR

Total DNA was extracted in the same manner as described by Mullis.[19] 16s rRNA gene was detected by PCR using specific primers (5'-CCA GCA GCC GCG GTA ATA CG-3' and 5'- ATC CGY TAC CTT GTT ACG ACT TC-3'). PCR reactions were performed in a 50 µL volume comprising 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 µg/mL of each primer, 1.5 U Taq DNA polymerase and 0.2 mM dNTP mix. The PCR conditions consisted of a predenaturation step at 94-degrees Celsius for 10 minutes, followed by 30 cycles of 1 min at 94 degrees, 40 seconds at 63 degrees, and 40 seconds at 72 degrees. A final extension step was performed at 72 degrees for five minutes.

The amplified products were analyzed by electrophoresis on 1.5 percent agarose gel, and the DNA bands stained with ethidium bromide, were photographed under ultraviolet illumination. The results from the coronal leakage were analyzed using Kaplan-Meier survival analysis. All analyses were performed by the Statistical Package of Social Science (SPSS Inc., Chicago, Ill.) for Windows version 13.

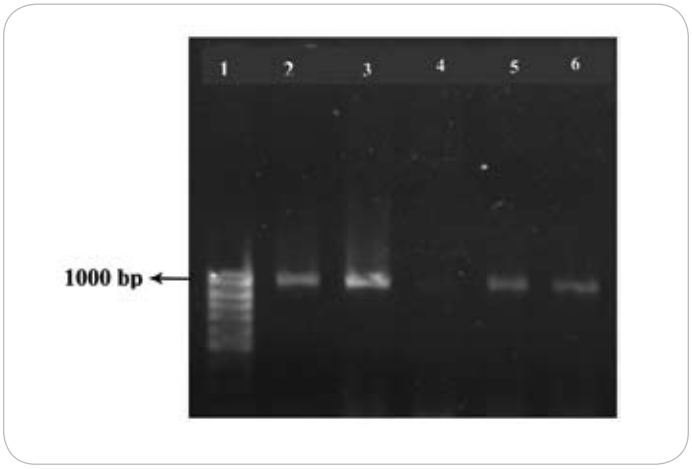

FIGURE 3. The image of PCR products by 16S rRNA gene on electrophoresis gel.

Results

One of the specimens was discarded because of the appearance of some cracks in part A in the first days of the experiment. The results revealed that seven (33 percent) teeth did not cause broth turbidity within 120 days. The time to leak in 14 leaked teeth is presented in **FIGURE 2**.

The time of contamination ranged from five to 110 days. The median time to leakage was 29 days (CI 95 percent, 0 to 93 days). In this period, 11 teeth leaked (79 percent of all leaked teeth in the study duration). Twenty-five percent of these teeth caused broth turbidity within eight days.

All the roots in the positive control group caused broth turbidity within 24 hours. In contrast, the broth in the negative control group remained clear throughout the experimental period. The broth with negative growth, inoculated with saliva from part C, showed turbidity within 24 hours. **FIGURE 3** shows the result of leakage-free teeth PCRs. It demonstrates there was no contamination in the last 3 mm of those teeth.

Discussion

One of the main principles of successful root canal treatment is the prevention of microorganisms and toxins from the oral flora penetrating through the root canal into the periapical tissues. This is achieved by complete filling of the root canal system, including the coronal and apical ends, using materials (sealers) that will prevent proliferation and/or diffusion along the interface between root canal surface and filling material, and through the root filling. Information on the rate of contamination of root canals would be useful for clinicians trying to decide whether or not to revise existing root fillings that have been exposed to the oral environment, e.g., by loss of the coronal restoration.

The ability of various root filling techniques and materials to prevent both coronal and apical microleakage (so-called "sealability") has been evaluated by many in vitro methods based on the assessment of the penetration of various tracers along filled root canals. The tracers most often used are dyes, radioisotopes, or bacteria.[20] However, although isotopes and dyes may be good tools for comparing relative leakage, they do not simulate the types of microbial leakage that may occur clinically.[21,22] In addition, the media used in such studies lack the proteins, enzymes, bacterial populations and their byproducts, which are normally found in natural saliva.

Subsequently, several authors have questioned the relevance of leakage studies in general, citing wide variation in results despite similar methodologies, poor (absent) controls, and no evidence of a link between leakage and presence of disease.[23,24] Even in those studies where bacteria have been used as tracers, it is noteworthy that the species used were not necessarily regarded as representative of bacteria playing a key role in root canal recontamination.[4,8,15,17]

The model used in this study was accurate, simple and practical; initial problems with leakage at the tooth/part B and tooth/plastic interfaces was resolved in two pilot studies. The main advantage of the present experiment over previous studies seeking to assess the time required for bacterial leakage to cause coronal leakage is the employment of natural saliva in anaerobic conditions.[4,8]

Consequently, it is considered that findings are more realistic assessment of the rate of leakage along root fillings in the coronal-apical direction.

Where attempts have been made to quantify the amount of leakage, some authors have suggested it is necessary to remove entrapped air from the obturated root canals, although others have questioned this view.[25-27]

Since, in the present study, the authors were measuring only the presence or absence of leakage, and because the removal of entrapped air from root fillings is not a practice attempted in the clinical environment, no attempt was made to remove entrapped air from the specimens.

The results of this study are similar to those reported by Khayat et al., who found that the median time to contaminate canals obturated, with a lateral compaction technique was 30 days, as compared with 29 days in the present study.[2] It is possible that the additional assessment of contamination by anaerobic bacteria explains the shorter time for leakage to be observed. Some teeth did not leak throughout the study. Presumably, aspects of the root canal structure and subsequent root filling in these teeth lead to this ideal observation, although it

is possible that leakage would have been observed if they had been kept longer than the 120 days of the experiment.

The results of the present study suggests that canals that have been exposed to the oral cavity for more than 10 days should be retreated. In vivo animal studies simulating the clinical situation are suggested in order to determine the exact rate of bacterial leakage in unsealed and exposed root filled teeth because in vitro studies are inherently limited by the lack of any host response to the microbial challenge.

Conclusions

Under the conditions of this study a third of all root canals with 10 mm length of root filling were recontaminated in less than 10 days. ■■■■

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