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An investigation of a potential confounder in *ex vivo* microbiological studies – the bulk flow of fluid through apical foramina during tooth extraction

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Abstract

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Aims To investigate the factors affecting bulk flow of dye and bacterial suspensions into and out of apical foramina during simulated tooth extraction, using an *ex vivo* model.

Methodology Sixty extracted, single-rooted, human teeth were accessed, root canals located and in 50 the pulps dissolved; 10 teeth with attached periapical lesions were preserved. The size of apical foramina was determined digitally. The teeth were mounted in vials with polyvinylsiloxane impression material. Part 1: different dyes were inoculated in the coronal half of root canals or cervical 'gingival' margin, respectively, in separate experiments using the same teeth. Tooth extraction movements were simulated and apical penetration of the dye solutions with and without coronal restorations were examined in each case (20 teeth reused 4 ×). Part 2: the same procedures were repeated on 30 more teeth but using a standard inoculum of *Acidovorax* sp. Part 3: 10 teeth with attached periapical lesions were inoculated with *Acidovorax* sp. in the absence of coronal restorations. Bacterial leakage into the periapical lesions was assessed.

Results Coronal restorations significantly reduced the flow of dyes ($P = 0.002$) or bacterial suspension ($P = 0.001$) out of the canals and bacterial suspension into ($P = 0.02$) the canals during simulated tooth extraction. The 'size of apical foramina' were positively correlated with passage of bacterial suspension out of the canal ($P = 0.04$) and from the gingival trough into the canal ($P = 0.008$), in the presence of a coronal restoration.

Conclusions The presence of coronal restorations, the size of apical foramina and presence of native canal contents with attached periapical lesions, all influenced fluid flow into and out of canals during simulated tooth extraction movements.

Keywords: fluid flow in canals, microbial displacement, root canal, tooth extraction.

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Introduction

Bacteria have long been established as the main aetiological agents of pulpal and periapical disease (Kakehashi *et al.* 1965) and their diversity explored both by culture (Bergenholtz 1974, Sundqvist 1976) and culture-independent techniques (Munson *et al.* 2002). However, the exact composition and topography of the root canal (Thilo *et al.* 1986, Nair 1987, Molven *et al.* 1991, Dougherty *et al.* 1998, Rolph *et al.* 2001, Richardson *et al.* 2009) and periapical (Boyle 1934, Sundqvist & Reuterving 1980, Nair & Schroeder 1984, Happonen *et al.* 1985, Happonen 1986, Sjögren *et al.* 1988, Barnett *et al.* 1990, Sen *et al.* 1995, Abou-Rass & Bogen 1998, Gatti *et al.* 2000, Sunde *et al.* 2000) microbiota have yet to be fully explored.

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Microbiological studies on root canal infections are biased by sampling, transport, cultivation and identification techniques (Gulabivala 2004). One of the controversies centres on the true location of bacteria in root canals and in periapical lesions when extracted teeth or biopsy samples are used as clinical samples for microbiological or histological studies. It has been hypothesized that the 'pumping action' induced during tooth extraction or sample retrieval, may transpose bacteria from the canal lumen to the periapex (Dahlén & Möller 1992, Walton & Ardjmand 1992) and *vice versa*. The early experimental work by Sir Wilfred Fish (Fish & MacLean 1936, Fish 1939) suggested that pumping may force bacteria originating from the gingival sulcus towards the periapical tissues either through a bacteraemia or by direct translocation. Only when the gingival sulcus was cauterized with hot wires, did the investigators fail to recover any bacteria from the periapical tissues (Fish 1939). Microorganisms may also be transposed from the canal lumen to the periapex (Walton & Ardjmand 1992). Bacterial masses have been demonstrated just beneath or slightly extruding from the apical foramen in histologic sections, even when teeth were removed *en block* (Nair 1987, Walton & Ardjmand 1992). These can easily be forced periapically during tooth extraction and thus be perceived as already present in the periapical tissues, leading to inaccurate conclusions about the presence and distribution of bacteria in and around tooth apices.

The extent to which such events may influence the validity of microbiologic studies has not yet been determined. It is likely that biofilm-encased bacteria adherent to the canal wall (Nair 1987) would not be translocated by fluid movement but those bacterial cells suspended in a fluid medium within the canal lumen (Nair 1987) could be so translocated by mass fluid flow induced by pressure differentials generated by the pumping action of tooth extraction. The discipline of fluid mechanics adopts numerous definitions and parameters to describe fluid flow, which may be complex in the root canal system but certainly remains, undescribed thus far (Gulabivala *et al.* 2010). The description of 'bulk flow' adopted here denotes translocation of a bolus of fluid from one part of the canal to the apex, without stipulating the nature, 'fluid-net' conditions (particles in different parts of the fluid volume may display unique vectors of motion) or complexity of flow.

The aim of this *ex vivo* study, based on preliminary work (Kapalas *et al.* 2001, 2002) was to investigate whether the pumping action employed during simulated tooth extraction may induce translocation of suspended bacteria by bulk fluid flow from the root canal to the periapical 'tissues' or in the opposite direction via the apical foramina; and to identify factors that could affect such phenomena.

Materials and methods

This *ex vivo* study was conducted in three parts and consisted of investigation of the: (i) bulk flow of dyes from the root canal system to the external root surface and vice versa in teeth without attached periapical lesions; (ii) bulk flow of bacterial suspension from the root canal system to the external root surface and vice versa in teeth without attached periapical lesions; and (iii) flow of bacterial suspension from the root canals of extracted teeth into attached periapical lesions.

Sample collection and storage

Sixty extracted, single-rooted, human, mature permanent teeth were collected from patients attending the Oral and Maxillo-facial Surgery Department, Eastman Dental Hospital, with informed consent. Teeth without attached periapical lesions or those with lesions dissected off were used in the first two parts of the study ($n = 20$, $n = 30$, respectively) and were immediately placed in 4% formal-saline solution for storage. Those teeth with attached periapical lesions were reserved for the third part of the study ($n = 10$) and following extraction were immediately placed separately in sterile vials (Bibby Sterilin Limited, Stone, UK) without rinsing or preserving solutions and were stored at −70 °C until processing.

Part 1 – Investigation of the bulk flow of dyes

Twenty extracted single-rooted teeth without attached periapical lesions were randomly selected for use by the toss of a coin; all 20 teeth were re-used for the four sets of experiments in this part, amongst which statistical comparisons were later made. The size of the apical foramina were estimated using photomicrographs of the apices obtained using a light microscope (Stemi 2000 Stereomicroscope, Zeiss Gruppe, Jena, Germany) at a standard (5×) magnification. The photomicrographs were scanned (Kodak RFS 3570 Professional Slide Scanner, Eastman Kodak Company, Rochester, NY, USA) and digitized. The cross-sectional area of the apical foramina was measured using an image analysis software package (Image Pro® Plus Version 4, Media Cybernetics, Silver Spring, MD, USA).

Following preparation of standard access cavities and location of the root canals, the teeth were immersed in 3% sodium hypochlorite overnight to dissolve pulp tissue remnants. The teeth were subsequently cleaned with sterile water in an ultrasonic bath. The experimental teeth were mounted in plastic vials using unset polyvinylsiloxane impression material (Extrude Light Body; Kerr Corporation, Romulus, MI, USA). Following setting of the impression material the tooth/impression interface was sealed with the recommended impression adhesive.

Assessment of dye leakage from the root canals outwards

A standardized volume (10 μL) of Crystal violet dye (Difco, Becton Dickinson Microbiologic Systems, Sparks, MD, USA) was deposited as a bolus into the coronal half of the root canals, where it remained filling the entire circumference of the canal. The access cavity was then sealed with an IRM® restoration. Tooth extraction movements consisting of apical and lateral movements were simulated using a pair of number 74 premolar extraction forceps (Dentsply Ash® Instruments, Plymouth, UK). The leakage of Crystal violet dye (violet colour) from the root canal to the external root surface was examined macroscopically and microscopically by virtue of the evidence of the presence of the dye. The threshold for the outcome measure was the presence of dye on the root surface surrounding the immediate vicinity of the apical foramen; the *amount* of dye leakage was not quantified.

The teeth were then removed from the plastic vials and cleaned by immersion in 3% sodium hypochlorite in an ultrasonic bath to remove the dye; this was followed by washing in sterile water in similar manner. The teeth were repositioned in vials containing polyvinylsiloxane impression material and the same experiment was repeated, this time in the absence of a coronal restoration.

Assessment of dye leakage from the gingival trough into the root canals

Following cleaning of the experimental teeth as described, the procedures were repeated with the application of Safranin dye (red colour) (Difco, Becton Dickinson Microbiology Systems) at the CEJ level in a marginal trough created within the simulated rubber base gingival margin. Leakage of Safranin dye from the external root surface along the length of the root to the apical foramina and into the root canals of the experimental teeth was examined in the presence of a coronal restoration (the first set of experiments). The experiments were repeated in the absence of a coronal restoration (second set of experiments) after removal of the dye as described previously. The presence of Safranin dye around and inside the apical foramen (the threshold for the outcome measure) was examined under the microscope as previously described. The experiments again generated two sets of data; one set for teeth with the coronal restorations (n = 20) and another set for the same teeth without the coronal restorations (n = 20).

Part 2 – Investigation of the flow of bacterial suspension into and out of teeth without attached periapical lesions

Preparation of bacterial suspension

The *Acidovorax* species (straight to slightly curved rods, 0.2–07 × 1.00–5.00 μm), isolated from a dental-unit water line was selected because of its easily recognizable bright orange colonies. The bacterial strain was received as frozen pure cultures in Brain Heart Infusion with Glycerol (BHIG) (Brain Heart Infusion-Lab M Ltd, Bury, UK; plus 10 v/v Glycerol – BDH Laboratory, Poole, UK). After defrosting, the isolate was cultured and R2A agar (Difco, Becton Dickinson Microbiology Systems) under aerobic conditions at 30 °C for 2 days. A dense suspension of the *Acidovorax* culture was prepared in 750 μL of reduced transport fluid (RTF).

Assessment of bacterial leakage from the root canals outwards

The flow of *Acidovorax* suspension through the apical foramen of teeth during simulated tooth extraction was studied using another 30 randomly selected teeth (randomization achieved by the toss of a coin) as described previously; the same set of 30 teeth were reused for four sets of experiments amongst which statistical comparisons were made. The cross-sectional area of the apical foramina was determined as previously described. Standard access cavities were prepared with root canal location but absence of mechanical preparation. The teeth were immersed in a container with 3% sodium hypochlorite in an ultrasonic bath to dissolve pulp tissue remnants and then cleaned with sterile water. The experimental teeth were then mounted in polyvinylsiloxane as previously described. An inoculum of *Acidovorax* sp. (10 μL) was pipetted into the coronal half of the root canal system as a bolus, where it remained filling the entire circumference of the canal. The access cavity was then sealed with an IRM® restoration. The experimental teeth were manipulated for extraction from their artificial sockets as previously

described. Three sterile paper points (Roeko, Langenau, Germany) wetted with sterile RTF were used to sample in a 'criss-cross' motion the external root surface surrounding the apical foramina. The samples were placed into sterile 1.5 mL Eppendorf tubes containing 750 μL of RTF, vortexed, plated out on R2A agar plates and incubated at 30 °C in aerobic conditions for 48 h. Leakage of *Acidovorax* from the root canal to the external root surface was determined by orange bacterial growth in the R2A plate cultures; the threshold for a positive outcome was a single orange colony.

The experimental teeth were cleaned by immersion in 3% sodium hypochlorite solution agitated in an ultrasonic bath to decontaminate the teeth, followed by similar washing in sterile water; absence of bacterial growth was confirmed by sterility checks performed by sampling as described before. The experiments were then repeated without the coronal restorations.

Assessment of bacterial leakage from the gingival trough into the root canals

The cleaning procedures were again repeated with sterility checks, followed this time by repetition of the experiments, by pipetting the *Acidovorax* inoculum into a marginal trough within the simulated rubber base gingival margin, as described previously. Leakage of the bacterial suspension along the root surface towards the apex and into the apical foramina was assessed following the performance of the previously described extraction movements. The experiments were performed in the presence and then separately in the absence of the coronal restorations following decontamination as described above on the same teeth.

Part 3 – Investigation of the flow of bacterial suspension out of teeth with attached periapical lesions

In the last part of the study, ten teeth with native canal contents and attached periapical lesions were used. The experimental teeth stored at −70 °C were thawed and used one at a time to minimize decomposition of the lesions and canal contents during the experimental procedures. The teeth were prepared as previously described and mounted in polyvinylsiloxane impression material by gently syringing around the teeth to prevent pressure on the lesions. The tooth/material interface was again sealed with an impression adhesive. A 10 μL inoculum of *Acidovorax* was pipetted as a bolus in the coronal half of the root canal system and tooth extraction simulated in the absence of a coronal restoration. Following extraction of the teeth from the simulated sockets, the periapical lesions were dissected with a sterile no. 15 scalpel blade, suspended in 1.5 mL Eppendorf tubes containing 750 μL of RTF, plate cultured on R2A agar and incubated at 30 °C in aerobic conditions. Leakage of bacterial suspension into the periapical lesion was confirmed by orange bacterial growth from samples obtained as described previously.

Analysis of findings

The McNemar test was used to compare the prevalence of dye or bacterial leakage between teeth with or without coronal restoration. The independent samples *t*-test was used to compare the cross-sectional area of the apical foramina between teeth with or without evidence of dye/bacterial leakage.

Results

Part 1 – Bulk flow of dyes

The data for this part of the study are presented in Tables 1 and 2.

The results indicated that the frequency of Crystal violet leakage out of the root canal through the apical foramen was higher in the absence of a coronal restoration (90% of teeth or 18/20) than when an intact IRM restoration was present (30% of teeth or 6/20). The McNemar test showed that the difference (60.0%; 95% CI: 29.4%, 90.6%) was significant ($P = 0.002$) (Table 1).

Safranin dye applied to the external root surface exhibited leakage into the root canal system in 55% of the teeth without a coronal restoration (11/20) but only in 35% of the teeth with intact coronal restorations (7/20). The McNemar test revealed that the difference (20.0%; 95% CI: −11.3%, 51.3%) was not statistically significant ($P = 0.3$) (Table 1).

Table 1 Number of teeth with and without dye translocation (leakage) out of or into the canal system by the presence and absence of coronal restoration

Experiment	Presence of coronal restoration	No leakage	Leakage	Total
Translocation of dye out of the canal	Yes	14	6	20
	No	2	18	20
Translocation of dye into the canal	Yes	13	7	20
	No	9	11	20

Table 2 Cross-tabulated data for association between cross-sectional area of apical foramina and dye leakage into and out of the canal system in the presence and absence of coronal restorations (n = 20)

		Mean area, μm^2 (SD)			
		Teeth with leakage	Teeth with no leakage	Mean difference (SE)	*P* values
Leakage out of root canal	Presence of coronal restoration	117669.2 (65207.6)	77999.5 (65360.8)	39669.7 (31872.0)	0.2
	Absence of coronal restoration	97204.6 (65856.9)	24162.5 (8456.3)	73042.1 (47727.0)	0.1
Leakage into root canal	Presence of coronal restoration	140070.6 (76174.3)	62885.7 (42312.6)	77184.9 (26218.6)	0.04
	Absence of coronal restoration	107951.2 (67338.5)	67838.2 (61335.6)	40113.1 (29098.2)	0.2

Statistical analysis with the Independent Samples *t*-test showed that the cross-sectional area of the apical foramina had a statistically significant (P = 0.04) effect on dye leakage only in the case of leakage into the canal in the presence of a coronal restoration (Table 2).

Part 2 – Bulk flow of bacterial suspension

The data for this part of the study are presented in Tables 3 and 4.

Only 30% of the teeth (9/30) gave positive bacterial cultures in samples taken from the external root surface from canal-inoculated bacteria in the presence of a coronal restoration. In the absence of the coronal restoration, 80% of the teeth (24/30) gave positive cultures from the external root surface. The presence of a coronal restoration had a statistically significant (difference = 50.0%; 95% CI: 28.8%, 71.2%; P = 0.001) effect on the leakage of bacterial suspension out of the root canal system (Table 3).

Bacterial inoculum from the marginal gingival trough on the external root surface leaked into the root canal system in 50% of the teeth (15/30) with a coronal restoration. However, in the absence of an intact coronal restoration 73% (22/30) of the teeth gave positive cultures. The McNemar test showed that the presence of a restoration had a statistically significant (difference = 23.3%; 95% CI: 4.9%, 41.8%; P = 0.02) effect on translocation of bacteria by fluid flow into and out of the root canal (Table 3).

Table 3 Number of teeth with and without bacterial translocation (leakage) out of or into the canal system by the presence and absence of coronal restoration

Experiment	Presence of coronal restoration	No leakage	Leakage	Total
Translocation of bacteria out of the canal	Yes	21	9	30
	No	6	24	30
Translocation of bacteria into the canal	Yes	15	15	30
	No	8	22	30

The independent samples *t*-test showed that the area of apical foramina had a positive correlation with bacterial translocation through them (Table 4).

Part 3 – Bulk flow of bacterial suspension in teeth with periapical lesions

Three out of ten experimental teeth with attached periapical lesions gave positive cultures from the external root surface, when bacteria were inoculated in the coronal half of the root canal system, which had not been previously cleared of native canal contents and contained no coronal restorations. No analytical statistical test was performed for this data set.

Discussion

The central problem with simulation models is universal; that of the proximity of test conditions to the native state being simulated. The histological state of healthy or inflamed pulp is of course well known but the nature of the infected root canal system has not been explored extensively (Nair 1987, Richardson *et al.* 2009). Available evidence suggests that the root canal system contents may be diverse depending on the health-disease continuum and that much of the resident tissue or bacterial biofilm is attached to the dentine wall in a manner which is difficult to displace through hydrodynamic shear stresses alone. There is, however, an inference that the infected root canal system contains fluid with suspended bacteria (Nair 1987, Richardson *et al.* 2009); the displacement of this fluid into the periapical tissues during tooth extraction is the subject of the present investigation. In addition, bacteria suspended in saliva may also be displaced into the alveolar socket during tooth extraction and thence into the periapical tissues and root canal system (Walton & Ardjmand 1992). This study attempted to simulate the hydraulic movement of fluids in and around the root and root canal system during tooth extraction. The exact conditions of ruptured connective

Table 4 Cross-tabulated data for association between cross-sectional area of apical foramina and bacterial inoculum leakage into and out of the canal system in the presence and absence of coronal restorations (n = 30)

		Mean area, μm^2 (SD)			
		Teeth with leakage	Teeth with no leakage	Mean difference (SE)	*P* values
Leakage out of root canal	Presence of coronal restoration	119917.4 (35005.0)	64696.5 (16377.5)	55221.0 (9272.6)	0.001
	Absence of coronal restoration	87917.8 (34693.8)	54642.7 (16608.8)	33275.1 (14705.3)	0.03
Leakage into root canal	Presence of coronal restoration	101483.1 (35555.8)	61042.5 (17754.9)	40440.6 (10261.4)	0.001
	Absence of coronal restoration	90514.5 (33593.3)	55820.6 (22734.3)	34693.8 (12895.5)	0.01

tissue fibres and blood vessels resulting in bleeding and bacteraemias could not be reproduced in this *ex vivo* model. Tooth extraction takes place in a fluid-saturated environment; an artificial socket had to be created for the purpose of this study using a material that would: (i) simulate the close adaptation and resilient characteristics of the alveolar tissues and (ii) allow preservation of the integrity of periapical lesions and their recovery for the third part of the study. Based on these criteria a light-body polyvinylsiloxane impression material constrained by a plastic container was selected after pilot studies. The tooth/impression material interface was sealed with an impression adhesive to provide shear resistance, mimicking the periodontal ligament as the tooth was manipulated for extraction. This gave a resilient yet adequately rigid mechanical approximation of the tooth to the associated 'socket'.

The experimental teeth used in the first two parts of the study were all intact, non-carious and un-restored, to ensure that any pressure differentials generated by tooth movement were not exhausted through structural flaws in the teeth. The teeth were observed under the microscope and only those with an absence of obvious signs of extensive apical resorption and a single apical foramen were selected. The first two parts of the study used the worst case-scenario, that of a canal system devoid of its native content and absence of an attached periapical lesion. The final, third part of the study retained the native canal contents and had firmly attached periapical lesions. The use of dyes enabled the tracking of the fluid movement; different dyes being used to separate out the possibility of confusion between tracking the movement of fluid from canal outwards and that in the reverse direction. The use of suspended bacteria mimicked the case of the subject under evaluation; that of translocation of planktonic bacterial cells along with the bulk fluid flow.

The root canals were located but left unprepared to mimic the clinical environment of native canal shape and surface in teeth with apical periodontitis. Pilot studies determined that repeated cycles of autoclaving had a detrimental effect on the physical and mechanical properties of dentine, as the teeth tended to fracture during extraction movements. The alternative strategy of immersion in 3% sodium hypochlorite to decontaminate teeth and neutralize the dyes proved effective and workable.

Measurement of the cross-sectional area of the apical foramina was considered important to account for potential confounding; however, measurement of an opening that lies on non-linear planes would always prove a challenge. In the final analysis, to minimize distortion and inaccuracy, the apical foramina were imaged at $5\times$ magnification perpendicular to their principal plane. Comparable data were not available in the literature but stereomicroscopic and SEM studies of root apices of anterior teeth (Green 1957, Morphis *et al.* 1994) suggested a diameter range of 0.4–1.00 mm (equivalent to 0.126–0.785 mm^2).

The bacterial species used in the second part of the study (*Acidovorax* sp.) was selected on the basis of its distinctive bright orange colonies, to serve easy identification. The species, isolated from biofilms in dental unit water lines, has never been recovered from the oral cavity or root canal systems of human teeth. Although capable of forming biofilms, the role of the bacterium in this study was to act as a cell suspension (in planktonic phase) and to be robust enough to survive and be distinct enough from the native microbiota for uncomplicated identification, particularly for the third part of the study. RTF was used as a medium for all the sampling procedures, as it has been shown to maintain the viability of bacteria (Syed & Loesche 1972), including the selected species.

The results of the first two parts of the study indicated with some clarity that the presence of an intact coronal restoration (compared to absence of a coronal restoration or patent canal system) significantly altered the fluid dynamics within and around root canals, reducing the flow of both dyes (fluid) and bacterial suspensions (particulate matter). In the third part of the study it was decided to test the leakage of a

bacterial suspension from the root canals of teeth containing their native contents towards firmly attached periapical lesions in the absence of a coronal restoration, in order to examine the worst case scenario.

The results of this study showed that the 'pumping action' inherent in conventional tooth extraction may cause bulk flow of dyes from the root canal and the marginal tissues towards the periapical lesion. The pressure differentials existing between the ambient atmosphere and the root canal system during tooth manipulation have the capacity to induce fluid movement. An intact coronal restoration (and tooth) created a 'closed pressure environment' and significantly ($P = 0.002$) reduced dye leakage out of the root canal system by 50–60%. However, it had no statistically significant effect ($P = 0.3$) on the leakage of Safranin dye from the cervical aspect of the external root surface towards the root canal system, where the reduction was of the order of 20–30%. Therefore, although not significant, the trend was the same. The effect of a coronal restoration was corroborated by the statistically significant effect on the leakage of bacterial suspension both into ($P = 0.02$) and out of ($P = 0.001$) the root canal system. The difference in the result between flow of dyes and bacteria into the canal from the gingival margin may reflect a higher sensitivity of the bacterial outcome measure.

The size of the apical foramen only had a significant effect on the flow of dye into the root canal system, in the presence a coronal restoration. In contrast, the size of the apical foramen had a significant positive association with the leakage of bacteria into as well as out of the root canal system regardless of the presence of a coronal restoration. The discrepancy between the effect of size of the apical foramen on the leakage of dye and on the leakage of bacteria may be related to differences in flow characteristics of the fluid suspensions. The inference is that the pressure differentials existing in the absence of a coronal seal are more likely to be such as to impel fluid movement through the apical foramina regardless of their size. In contrast, the presence of a coronal seal (or intact tooth) creates a pressure system that is more dependent upon the diameter of the apical foramina for fluid motion events.

Three out of ten teeth with intact native canal contents and firmly attached periapical lesions gave positive cultures of the test organism from the periapical lesions in the absence of a coronal restoration. Comparing these results with the outcomes of parts 1 and 2, it would be anticipated that if a significant volume of fluid with suspended planktonic bacteria were present in the root canal system, then their outward flow would have been anticipated. Microscopy studies (Nair 1987, Richardson *et al.* 2009) offer some insight into the canal contents, where in addition to surface adherent bacterial biofilms, which may narrow the canal gauge, the periapical lesion was also sometimes separated from the canal by a wall of polymorphonuclear leukocytes or proliferating epithelial plugs. Mixed bacterial clusters were also observed within the epithelial plug and along the epithelium-dentine interface. Although the epithelial plug was infrequently observed and wide intercellular spaces were always present (Nair 1987), the collective biologic barrier in the canal and apex could reduce the leakage of fluids both into and out of the root canal system during extraction. This last part of the study evaluated the bulk flow transference of the bacterial inoculum and did not account for any extrusion of the previously resident canal contents during extraction manipulation.

The penetration of bacteria from the marginal attachment into the lesion was not examined in this part of the study as the teeth could not be re-used following lesion dissection. Tooth extraction forces may however pump bacteria originating from saliva or plaque towards the periapical lesion. Human periapical lesions consist of granulomatous tissue, infiltrating immune cells, fibroblasts and a well-developed fibrous tissue capsule which, when present is firmly attached to the root surface with dense collagenous fibres (Nair 1997). The degree to which this tissue can present a barrier to bacterial penetration has not been determined. It is likely that only lesions with firm fibrous barriers would be amenable to extraction attached to the associated tooth; compared to lesions in which the fibrous capsule is poorly developed or has been destroyed by suppuration. The sample of extracted teeth with attached lesions may therefore be considered biased, however, they are also most likely to be the *true* chronic lesions with absence of suppuration that merit study for extra-radicular infections. It is already well established that acute lesions contain bacteria (Nair 1987).

The present study indicated that the 'pumping action' induced during tooth extraction can cause movement of fluid-suspended bacteria into and out of the root canal system, under certain conditions. The existence of open communication between the root canal system and the oral cavity, as in cases of

profoundly carious teeth, was shown to increase the risk of bacteria being forced through the apical foramen during extraction. Carious teeth have been almost exclusively used in the study of the composition and topography of the root canal flora (Nair 1987, Baumgartner & Falkler 1992, Siqueira & Lopes 2001, Siqueira *et al.* 2002). Based on the results of this study, the topography of the intra- and extra-radicular microflora could have been altered as a result of the extraction procedure. It could be suggested that an intact pulp chamber or a coronal restoration or a root-filled tooth could potentially decrease the risk of fluid flow into and out of the root canal, however, it has been claimed that even in cases of tooth removal in block sections (Nair 1987), bacterial colonies could extrude out of the apical foramen without evident tissue disruption (Walton & Ardjmand 1992). The authors claimed to have made the same observation in their own specimens (Walton & Ardjmand 1992) and that these bacteria could have easily been forced periapically if the teeth were extracted rather than removed *en block*. These suggestions were contradicted by the previously mentioned author who maintained that no such findings were present in any of his published or unpublished data (Prof. Nair, personal communication).

The anatomy of the apical foramen in its native or altered state (biological or iatrogenic) allows greater fluid flux when larger in diameter but the pressures are altered with greater or lesser influence from the diameter depending on the presence or absence of coronal integrity.

In addition to the fluid flux created by the pumping motion of the tooth in the socket, the flexure of the tooth (Paphangkorakit & Osborn 2000) or restoration in the cavity (Hirata *et al.* 1991) has also been implicated in micro-volume fluid movement within teeth. The former group measured fluid movement of 2–6 × 103 pL with a load of 2 kg to 13.2–34.8 × 103 pL with a load of 12 kg. Sealing the root canal openings eliminated or reduced such micro-flow. Kishen (2005) also demonstrated that cyclic loading of extracted teeth resulted in apical fluid movement into the root canal; to a depth of 3.8 mm in intact teeth, 6.1 mm in teeth with enlarged canals maintained wet and 2.1 mm in teeth with enlarged root canals maintained dry. It was postulated that such fluid may feed nutrients to apical microbiota but it was not clear whether such fluid motion would be capable of moving bacteria and altering their distribution; it seems unlikely (Gulabivala *et al.* 2010).

For microbiological or histo-microscopy studies on extracted teeth with periapical disease, it would be prudent to select intact teeth with intact pulp chambers or coronal restorations and to extract such teeth with minimal 'pumping' motion, preferably using perio-tomes or luxators to gently expand sockets first.

Conclusions

Within the limitations of this study the following conclusions can be drawn:

The presence of a coronal restoration or an intact tooth significantly reduced the flow of dyes ($P = 0.002$) and bacterial suspension ($P = 0.001$) out of the root canal system and the flow of bacterial suspension into ($P = 0.02$) the root canal system during simulated tooth extraction.

The size of the apical foramen had a positive association with translocation of bacterial suspension through the apical foramen only in the presence of a coronal restoration, both when bacteria were inoculated into the root canal system ($P = 0.04$) and when inoculated into the marginal gingival trough ($P = 0.008$).

Microbiologic/microscopic studies on root canal and periapical microbiota using extracted teeth should account for potential contamination due to bacterial translocation and minimize the risk according to factors identified.

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