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Impact of Growth Conditions on Susceptibility of Five Microbial Species to Calcium Hydroxide

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Key Words: Biofilm, calcium hydroxide, *Candida albicans*, dentin disinfection, *Enterococcus faecalis*, root canal

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Abstract

The effects of different growth conditions on the susceptibility of five taxa to calcium hydroxide were investigated. *Enterococcus faecalis* ATCC 29212, *Streptococcus sobrinus* OMZ 176, *Candida albicans* ATCC 90028, *Actinomyces naeslundii* ATCC 12104 and *Fusobacterium nucleatum* ATCC 10953 were grown as planktonic cells, allowed to adhere to dentin for 24 h, grown as mono-species or multi-species biofilms on dentin under anaerobic conditions with a serum-enriched nutrient supply at 37°C for 5 d. In addition, suspended biofilm microorganisms and 5-d old planktonic multispecies cultures were used. Microbial recovery upon direct exposure to saturated calcium hydroxide solution (pH 12.5) for 10 and 100 min was compared to control exposure to physiologic saline. Planktonic microorganisms were most susceptible, only *E. faecalis* and *C. albicans* survived in saturated solution for 10 min, the latter also for 100 min. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* and *A. naeslundii* to calcium hydroxide, while the multi-species context in a biofilm was the major factor in promoting resistance of *S. sobrinus* to the disinfectant. In contrast, the *C. albicans* response to Ca(OH)₂ was not influenced by the growth condition. Adherence to dentin and inter-species interactions in a biofilm appear to differentially affect the sensitivity of microbial species to calcium hydroxide.

Microorganisms commonly isolated from root canal infections are susceptible in planktonic suspensions to alkaline stress caused by calcium hydroxide *in vitro* (1). Even the most resistant species such as *Enterococcus faecalis* and *Candida albicans* survive only limited periods of time in the high pH of 12.5 of a saturated aqueous calcium hydroxide solution (1, 2). However, experimental eradication of *E. faecalis* from infected bovine dentin blocks has shown to be difficult (3). Most likely due to factors related to experimental infection depth, complete disinfection of extracted human premolars from *E. faecalis* by a calcium hydroxide dressing has recently been reported (4). On the other hand, a review on clinical trials has suggested limited efficacy of calcium hydroxide in eradication of microorganisms from infected root canals after instrumentation and irrigation with a sodium hypochlorite solution (5).

Due to the number of possible confounding factors associated with clinical trials, *in vitro* studies are essential for the development of efficient treatment strategies in root canal and dentin disinfection. During the past decades, a wide variety of different methodologies have been introduced and the obtained outcomes have sometimes been interpreted carelessly. Particularly, clinical conclusions based on *in vitro* data must be considered with care. Infected root canal systems usually contain several different cultivable taxa (6). Between microbes that can potentially invade the pulpless root canal, both, positive and negative interactions have been observed. In this context, understanding the growth of individual microbial species grown in different model systems appears to be important, as well as the impact of these conditions on survival of the microbes when they are exposed to an antiseptic (7). However, data on direct methodological comparisons in the endodontic literature is scarce.

The aim of this study was to compare the survival of five microbial species associated with endodontic infections under different experimental conditions. Upon an alkaline challenge induced by direct exposure to a calcium hydroxide solution, the recovery of

microbiota was compared between models employing single species in their planktonic form, single species adhered to dentin disks, and an established dentin biofilm comprising all five species.

Materials and Methods

Preparation of dentin disks

Standardized bovine dentin disks were prepared as follows: cylinders with a diameter of 7 mm were cut from the roots of extracted bovine front teeth using a trephine bur. Subsequently, a disk with a thickness of 0.8 mm was cut from the dentin section of the cylinder using a saw microtome (SP 1600, Leica, Wetzlar, Germany). The surface of the disk facing the outer root section was marked with a pencil. Disks were stored in sterile saline solution at 5°C until further usage.

Microorganisms

The following microorganisms were used in this study: *Actinomyces naeslundii* ATCC 12104, *Fusobacterium nucleatum* ATCC 10953, *Streptococcus sobrinus* OMZ 176, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 90028.

Planktonic bacteria

C. albicans and *E. faecalis* were grown on tryptic soy agar (TSA, Difco, Detroit, MI). The other three taxa were kept on Schaedler blood agar plates (Becton Dickinson GmbH, Heidelberg, Germany). Loopfuls of agar-grown cells were inoculated into fluid medium (Table 1). Pre-cultures (5 mL) of each species were incubated anaerobically at 37°C for 24 h (*C. albicans*, *E. faecalis*, *S. sobrinus*) and 72 h (*F. nucleatum*, *A. naeslundii*). Subsequently, microbial suspensions were washed and resuspended in saline. Cultures were adjusted to an

OD₅₅₀ of 1.000 ± 0.050 . The purity of the cultures was controlled by Gram-stained preparations and examination under a light microscope.

Adherent microorganisms

Microorganisms were grown and prepared as described above. 1.6 mL of each microbial suspension in saline was transferred into a 24-well cell culture plate (Bio Mérieux) containing a bovine dentin disc with the pulpal side facing up and incubated anaerobically at 37°C for 24 h. Subsequently, the discs were gently dipped three times in sterile saline in order to remove loosely adherent cells.

Monospecies biofilm

Single strains were allowed to form a monospecies biofilm in a similar set-up with dentin discs placed in 24-well cell culture plates. A protein film was obtained on the discs by incubating them in diluted horse serum (1/10 in saline) at 37°C for 2 h. The serum solution was then replaced with 1.6 mL of fluid universal medium (FUM, (8)) and inoculated with 100 µL of microbial suspension and incubated anaerobically at 37°C for 5 days. The culture media was refreshed after approximately 60 h of incubation. Prior to this and in the end of the 5 d incubation the discs were gently dipped and agitated three times in sterile saline in order to remove loosely adherent cells.

Five-species biofilm

The multispecies biofilm model used here is a modification of the model developed at the Institute of Oral Microbiology and Immunology in Zürich (9). Briefly, the species selection was adapted to endodontic interest by replacing *Streptococcus oralis* with *Enterococcus faecalis* ATCC 29212. *Veillonella dispar* was left out. In addition, the enrichment of FUM with glucose supplement was replaced by the enrichment with serum

more relevant to the conditions in an infected root canal. Prior to infection a protein film was obtained on the disks by incubation in diluted horse serum at 37°C for 2 h. The disks in serum-enriched FUM (1.6 mL in each well of the 24-well cell culture plates, BioMérieux) were inoculated with 100 µL of the mixed microbial suspension and incubated at 37°C for 5 days. Refreshing the broth and removal of loosely adhered cells was carried out as described above. All procedures and incubation were performed in a strict anaerobiosis using an anaerobic chamber with a gas mixture of CO₂ (10%), H₂ (10%), and N₂ (80%).

Resuspended microbiota from multi-species biofilms

To differentiate the effect of a three-dimensional structure of an established biofilm from stationary metabolic activity to susceptibility to calcium hydroxide, microorganisms grown in biofilms were suspended by a vigorous vortexing for 2 min followed by a gentle ultrasonication (20 W, 5 s, Vibracell, Sonics & Materials, Newtown, CT). Subsequently, the cells were washed and suspended in sterile saline as described above before their use.

Coaggregates

To control microbial interactions without a solid substrate for biofilm formation, multispecies cultures in FUM (1.6 mL) were incubated anaerobically at 37°C on an orbital shaker (100 rev/min) for 5 d. The nutrient broth was refreshed after 60 h of incubation. The obtained co-aggregates were washed in sterile saline and suspended as described above before their use.

Exposure to calcium hydroxide

One hundred µL of each strain of planktonic, suspended biofilm-grown as well as suspended coaggregate-associated microorganisms were exposed to 1.9 mL of saturated aqueous calcium hydroxide solution (pH 12.5) (Merck, Darmstadt, Germany). Similarly,

dentin disks with adherent microorganisms or biofilms were transferred to wells containing 2 mL of a saturated calcium hydroxide solution. Incubation in sterile saline was used as the positive control. After 10 and 100 min, disks were dipped and gently agitated three times in sterile saline. Each disk was transferred into a sterile 50 mL tube containing 1 mL of saline and vortexed vigorously for 2 min. This was followed by ultrasonication for 5 s. Serial dilutions of 10^{-1} to 10^{-5} in saline were prepared and aliquots of 10 μ L were plated onto agar plates listed in Table 3. Colonies were counted after 48-72 h of incubation at 37°C under a stereomicroscope. Their identification was based on growth conditions, colony morphology and cellular characteristics after Gram-staining (9).

Data presentation

The numbers of recovered microbiota are presented as \log_{10} colony-forming units (CFUs)/mL in test and control suspensions (planktonic growth, coaggregates, and resuspensions) or in corresponding suspensions obtained after harvesting the adherent microbiota from the dentin disks (adhered and biofilm conditions). Data are presented as means and standard deviations.

Results

In the control experiments (exposure to saline), absolute and relative numbers of planktonic, adherent and biofilm-associated CFU counts showed remarkable variation, i.e. not all species grew equally well under the different conditions (Table 2). The exception was *A. naeslundii* illustrated by similar counts regardless of the environmental parameters used in this study. However, throughout the study, data variance within the respective growth condition for the species under investigation remained low.

In general, planktonic microorganisms were most susceptible to the saturated calcium hydroxide solution, which eliminated all the species but *E. faecalis* and *C. albicans* in 10 min; only the latter survived in low numbers for 100 min. When microbiota were re-suspended after growing in a five-species biofilm context for five days, there was no apparent change in their survival upon exposure to calcium hydroxide compared to that of early stationary phase organisms. The same was the case with five-day cultures of coaggregates in suspension. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* and *A. naeslundii* to calcium hydroxide, while the multi-species context in a biofilm was the major factor in promoting resistance of *S. sobrinus* to the disinfectant. Multi-species biofilm formation also improved the survival of *E. faecalis* in comparison to planktonic or adherent cells. The difference compared to adherent organisms was not obvious at 10 min but appeared remarkable after 100 min of exposure. In contrast, the *C. albicans* response to Ca(OH)_2 was not influenced by the growth condition (Table 2).

Discussion

This study demonstrated that growth conditions differentially affect the response of the five microbiota under investigation to calcium hydroxide. Dentin adhesion was the major factor in improving the resistance of *E. faecalis* ATCC 29212 and *A. naeslundii* ATCC 12104 to calcium hydroxide, while the multi-species context in a biofilm was the major factor in promoting resistance of *S. sobrinus* OMZ 176 to the disinfectant. In contrast, growth conditions did not affect *C. albicans* ATCC 90028 recovery.

It should be realized that the current study was designed to compare different growth conditions of microbiota associated with endodontic disease and their impact on susceptibility of individual strains to an alkaline challenge. The multi-species biofilm model used in the current study was derived from an established six-species model that is used to test

disinfectants against cariogenic plaque (9, 10). The original model has been validated and proven to yield results comparable with the situation *in situ* when oral antiseptics were tested at their clinically applied concentration. Compared to the original model, *Veillonella dispar* and *Streptococcus oralis* were not used in the current investigation. Instead *E. faecalis* was introduced, as this species is believed to commonly survive topical antiseptics in the root canal (11). Root dentin rather than enamel disks were utilised to reflect the spatial situation in the root canal. As with the original model, easily accessible laboratory strains were used. This has the advantage that the current results can be repeated in any laboratory. On the other hand, clinical strains often bear virulence factors that are lost with their laboratory counterparts. Consequently, care should be exercised to extrapolate the current results to the clinical situation.

The present data regarding the increased resistance of most microbiota to an alkaline challenge in biofilm compared to planktonic form are in line with a multitude of publications (12). On the other hand, the results obtained with *C. albicans* presented here would contradict or at least put the commonly held paradigm that “biofilm” equals resistance in perspective. In comparison to adherent bacteria, well-established biofilms have a number of additional protective properties against antimicrobial agents. These properties include a biofilm matrix, altered growth rate of biofilm organisms, and other physiologic changes due to the biofilm mode of growth (12). It is a prerequisite for antimicrobial agents to diffuse through the biofilm matrix in order to inhibit or kill the encased cells. The extracellular polymeric substances constituting this matrix present a diffusional barrier by affecting either the rate of transport of the agents into the biofilm or inhibits the agents directly by binding and dilution. Another proposed mechanism for biofilm resistance to antimicrobial agents is that biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly (13). However, as shown in the current study, not all microbial species profit from the spatial biofilm environment to a similar extent. *C. albicans*,

for example, appeared to survive but not be protected in the biofilm. It is well known from clinical observations that there are both positive and negative microbial interactions in a close spatial context (14). The five species in the current model were chosen so that no complete inhibition of any taxon occurs, but at the same time this model might reflect the situation of microbial aggregates in the root canal in that not only positive interactions appear to take place between individual species.

Future studies using this model could address the impact of type and concentration of endodontic disinfectants on the survival of the individual species.

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TABLE 1. Maintenance of microorganisms prior to the experiments.

Species	Strain	Broth	Agar
<i>A. naeslundii</i>	ATCC12104	Thioglycolate bouillon (Oxoid GmbH, Wesel, Germany)	Schaedler blood agar with Vit. K and 5 % sheep blood (Becton Dickinson GmbH, Heidelberg, Germany)
<i>F. nucleatum</i>	ATCC10953	Thioglycolate bouillon	Schaedler blood agar with Vit. K and 5 % sheep blood
<i>S. sobrinus</i>	OMZ176	Thioglycolate bouillon	Schaedler blood agar with Vit. K and 5 % sheep blood
<i>E. faecalis</i>	ATCC29212	Tryptic soy broth (Oxoid)	Tryptic soy agar (Oxoid)
<i>C. albicans</i>	ATCC90028	Tryptic soy broth	Tryptic soy agar

TABLE 2. Susceptibility of five microbial species after different growth conditions to saturated aqueous calcium hydroxide solution (\log_{10} CFUs/mL; N=5).

Growth	Species	10 min				100 min			
		Saline		Ca(OH) ₂		Saline		Ca(OH) ₂	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Planktonic 24h	<i>E. faecalis</i>	8.28	±0.39	1.26	±1.16	8.66	±0.50	0.00	±0.00
	<i>C. albicans</i>	6.47	±0.15	6.18	±0.15	6.28	±0.38	2.50	±0.43
	<i>A. naeshlundii</i>	5.39	±0.25	0.00	±0.00	ND ¹	ND	ND	ND
	<i>F. nucleatum</i>	6.38	±0.21	0.00	±0.00	ND	ND	ND	ND
	<i>S. sobrinus</i>	6.85	±0.19	0.00	±0.00	ND	ND	ND	ND
Adherent 24h	<i>E. faecalis</i>	6.75	±0.26	5.62	±0.53	6.55	±0.22	2.68	±1.50
	<i>C. albicans</i>	5.74	±0.26	5.38	±0.31	5.27	±0.19	1.77	±1.39
	<i>A. naeshlundii</i>	3.28	±0.27	3.28	±0.33	3.68	±0.67	0.00	±0.00
	<i>F. nucleatum</i>	3.02	±0.15	0.00	±0.00	ND	ND	ND	ND
	<i>S. sobrinus</i>	5.19	±0.19	0.00	±0.00	ND	ND	ND	ND
Adherent 5d	<i>E. faecalis</i>	6.71	±0.33	6.67	±0.27	6.88	±0.39	5.44	±0.59
	<i>C. albicans</i>	3.86	±0.31	3.14	±1.79	3.84	±0.25	1.68	±0.66
	<i>A. naeshlundii</i>	5.33	±0.66	5.39	±0.74	5.28	±0.58	3.73	±0.55
	<i>F. nucleatum</i>	4.53	±0.38	0.00	±0.00	ND	ND	ND	ND
	<i>S. sobrinus</i>	4.41	±0.35	0.00	±0.00	ND	ND	ND	ND
Biofilm 5d	<i>E. faecalis</i>	5.97	±0.25	5.74	±0.46	5.97	±0.22	5.19	±0.21
	<i>C. albicans</i>	2.37	±0.33	1.51	±0.41	2.37	±0.27	0.45	±1.00
	<i>A. naeshlundii</i>	5.89	±1.07	4.21	±0.44	5.89	±0.60	3.57	±0.19
	<i>F. nucleatum</i>	4.01	±0.32	0.50	±0.70	4.01	±1.32	0.00	±0.00
	<i>S. sobrinus</i>	4.08	±0.17	2.80	±0.77	4.08	±0.26	2.92	±0.61
Suspended Biofilm 5d	<i>E. faecalis</i>	5.81	±0.17	0.00	0.00	5.78	±0.17	0.00	±0.00
	<i>C. albicans</i>	3.04	±0.24	1.98	±1.14	2.91	±0.41	0.34	±0.76
	<i>A. naeshlundii</i>	5.88	±0.16	1.60	±1.46	5.71	±0.50	0.00	±0.00
	<i>F. nucleatum</i>	2.61	±0.55	0.00	±0.00	ND	ND	ND	ND
	<i>S. sobrinus</i>	4.83	±0.23	0.00	±0.00	ND	ND	ND	ND
Suspended coaggregates 5d	<i>E. faecalis</i>	6.55	±0.22	0.00	±0.00	6.80	±0.14	0.00	±0.00
	<i>C. albicans</i>	5.57	±0.14	4.88	±0.34	5.90	±0.50	3.75	±0.00
	<i>A. naeshlundii</i>	5.01	±0.47	1.02	±0.67	4.94	±1.34	0.00	±0.00
	<i>F. nucleatum</i>	2.08	±1.37	0.00	±0.00	ND	ND	ND	ND
	<i>S. sobrinus</i>	3.94	±0.23	0.00	±0.00	ND	ND	ND	ND

¹ND, not done

TABLE 3. Growth conditions used after harvesting multispecies cultures.

Agar	Incubation	Period
Enterococcus BAA, Oxoid	In air	48 h
CAND Biggy, Oxoid	In air	48 h
Mitis-Salivarius, Difco	5% CO ₂ in air	48 h
Schaedler blood agar	Anaerobically	72 h