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Influence of Serum and Necrotic Soft Tissue on the Antimicrobial Effects of Intracanal Medicaments

Julio C. Machado de OLIVEIRA
Flávio R. F. ALVES
Milton de UZEDA
Isabela N. RÔÇAS
José F. SIQUEIRA JR.

Department of Endodontics, Dental School, Estácio de Sá University, Rio de Janeiro, RJ, Brazil

The purpose of this study was to investigate the influence of serum and necrotic soft tissue on the antimicrobial activity of intracanal medicaments. The medicaments tested were: calcium hydroxyde/glycerin paste, calcium hydroxide/chlorhexidine paste, calcium hydroxide/camphorated paramonochlorophenol/glycerin paste, and chlorhexidine/zinc oxide paste. Survival of *Enterococcus faecalis* and *Candida albicans* exposed to the medicaments tested in the presence or absence of serum or necrotic tissue was monitored in three *in vitro* experiments where samples for culturing were taken at different time periods. The overall results demonstrated that the antimicrobial activity of all intracanal medicaments tested was slowed down in the presence of necrotic tissue. Calcium hydroxide pastes in glycerin or chlorhexidine were significantly affected by serum. Of the medicaments tested in this study, the least affected was the calcium hydroxide/camphorated paramonochlorophenol/glycerin paste.

Key Words: *Enterococcus faecalis*, *Candida albicans*, intracanal medication, antimicrobial activity, serum, organic matter.

INTRODUCTION

The rationale for treatment of any infectious diseases is elimination of causative microorganisms. Given the ultimate importance of microorganisms in the etiology of apical periodontitis, endodontic procedures should be directed towards the elimination of occurring microorganisms and/or prevention of infection or reinfection of the root canal space (1).

Although chemomechanical procedures are highly effective in reducing bacterial populations within the main root canal (2,3), bacteria located in some areas of the root canal system, such as untouched canal walls, dentin tubules, isthmuses, lateral canals and apical ramifications, may still remain unaffected by instruments and irrigants and then jeopardize the treatment outcome (4). This is a result of the physical limitations of endodontic instruments and the short time irrigants remain in the root canal during instrumentation/irrigation procedures. Although sodium hypochlorite (NaOCl) has excellent antimicrobial activity against most putative endodontic pathogens (5), this irrigant is allowed to act within the root canal system only for a short period. As chemomechanical preparation procedures have been expedited after introduction of rotary instruments, the permanence of NaOCl within the root canal has been even shortened. It has been demonstrated that irrigation with NaOCl in different concentrations is not sufficient to render root canals free of bacteria in about one half of the cases (2,3).

Because intracanal medicaments are placed for longer periods within the root canal, they have more chances to reach microorganisms in those areas inaccessible to instruments and irrigants. However, for a given medicament to be effective in killing microorganisms in areas like isthmuses and ramifications, it has to be effective in the presence of necrotic soft tissue and tissue fluids. The antimicrobial activity of several endodontic medicaments has already been demonstrated to be reduced or inactivated by dentin, hydroxyapatite, collagen and serum proteins (6,7).

This study investigated the influence of serum

Correspondence: Prof. Dr. José F. Siqueira Jr, Faculdade de Odontologia, Universidade Estácio de Sá, Avenida Alfredo Baltazar da Silveira, 580/cobertura, Recreio, 22790-701 Rio de Janeiro, RJ, Brasil. Tel/Fax: +55-21-2497-8988. e-mail: jf_siqueira@yahoo.com; siqueira@estacio.br

and necrotic soft tissue on the antimicrobial activity of intracanal medicaments against *Enterococcus faecalis* and *Candida albicans,* two microbial species commonly found in persistent endodontic infections (8,9).

MATERIAL AND METHODS

The following agents were tested in the present study: calcium hydroxide/glycerin paste (CHG), CH/camphorated paramonochlorophenol (CPMC)/glycerin paste (CHPG), CH/0.2% chlorhexidine gluconate paste (CHCx), 0.2% chlorhexidine gluconate/zinc oxide paste (CxZO). All pastes were prepared by adding the powder (CH or ZO) to the liquid until a creamy consistency was achieved. In CHPG paste, CPMC and glycerin were used in a 1:1 ratio (vol:vol).

The antimicrobial activities of the medicaments in the presence of serum and/or necrotic muscle tissue were assessed in 3 experiments. Microbial suspensions used as inoculum for the experiments were prepared as follows. *E. faecalis* (ATCC 29212) and *C. albicans* (ATCC 10231) were grown aerobically for 24 h on trypticase soy agar or Sabouraud agar plates (Difco, Detroit, MI, USA), respectively. Colonies were harvested, suspended in 5-mL trypticase soy broth and adjusted to match the turbidity of a 0.5 McFarland $BaSO_4$ standard. Experiments were carried out as described next.

Experiment 1

Fresh minced bovine muscle was placed in flasks containing saline solution and left at room temperature for 2 months. Afterwards, minced muscle, hereafter referred to as "necrotic tissue", was autoclaved and tissue sets of 1 g each were mixed and incubated with 1 mL suspensions of either *E. faecalis* or *C. albicans* for 24 h at 37ºC. Afterwards, 1 mL of the prepared pastes was placed at the bottom of wells of 24-well cell culture plates (Corning Glass Works, Corning, NY, USA). Wells containing 1 mL of 0.85% sterile saline solution instead of antimicrobial medicament served as the control of microbial survival. A layer consisting of 1 g of wet contaminated necrotic tissue was placed onto the pastes with care so as to maximize direct contact without mixing. For control, 1 mL of culture of the test microorganism instead of tissue was poured into the wells containing pastes or saline.

The cell culture plates were placed inside sealed plastic bags and then incubated at 37ºC for different periods of time. After time intervals of 1 h, 1 day and 2 days, aliquots of 100 µL were taken from each well and transferred to tubes containing sterile saline solution for 10-fold serial dilution. Aliquots of each dilution were plated onto trypticase soy agar (for *E. faecalis*) or Sabouraud agar plates (for *C. albicans*) and incubated at 37ºC for 5 days. Further, colony-forming units (CFU) grown onto the plates were counted.

Experiment 2

In this experiment, necrotic tissue prepared as above was mixed with each paste (1 g of tissue for 1 mL of paste) and incubated for 24 h at 37ºC. Afterwards, the tissue-paste mixture was applied to the bottom of wells of 24-well cell culture plates and 1 mL of *E. faecalis* suspension was poured over the mixture. Wells containing 1 mL of 0.85% sterile saline instead of antimicrobial medicament served as control for bacterial survival. Controls with no necrotic tissue were done as in experiment 1. The cell culture plates were incubated at 37ºC for 10 min, 1 h, and 1 day.

Aliquots of 100 µL were taken from each well and transferred to tubes containing sterile saline for 10-fold serial dilution. Aliquots of each dilution were plated onto trypticase soy agar and were incubated at 37ºC for 5 days. Further, CFU grown onto the plates were counted.

Experiment 3

For this experiment, 1 mL of each paste or saline (control) was placed to wells of cell culture plates and incubated with 1 mL of fetal bovine serum (Seromed, Sorali Biotecnologia, Campo Grande, MS, Brazil) for 9 days. Serum was renewed every 3 days. Next, 1 mL of culture of either *E. faecalis* or *C. albicans* was added to each well. Controls consisted of saline instead of serum. Plates were incubated at 37ºC for 30 min, 1 h, 1.5 h, and 1 day. Aliquots of 200 µL were taken from each well and transferred to tubes containing fresh thioglycolate broth and incubated at 37ºC for 1 week. Presence of turbidity was indicative of microbial growth. Whenever turbidity was determined, purity of the cultures was checked by Gram-staining and morphology of colonies grown onto blood agar or Sabouraud agar plates.

All experiments were performed in duplicate. For experiments 1 and 2, where CFU were counted, values are expressed as the mean number.

RESULTS

Experiment 1

In this experiment, the necrotic tissue was contaminated prior to testing. No significant antimicrobial effects were observed for any medicament after 1 h. In the absence of necrotic tissue, no viability was observed for *E. faecalis* and *C. albicans* when in contact with the test medicaments for 1 day. In the presence of necrotic tissue, all medicaments also succeeded in eliminating *E. faecalis* cells after 1 day, except for CxZO, which reduced the number of bacterial cells, but was clearly negatively affected by necrotic tissue (Table 1). Similar effects were observed after 2 days of incubation. As for *C. albicans*, in addition to CxZO, CHG also had its antimicrobial effects hampered by the presence of tissue in the 1-day evaluation period (Table 2). After 2 days, however, viable *C. albicans* cells, while reduced in numbers, were found only for CxZO treatment. Controls for both *E. faecalis* and *C. albicans* placed in saline were positive for viability throughout the course of the experiment.

Experiment 2

In this experiment, medicaments were incubated with the necrotic tissue before adding *E. faecalis* culture. No significant antibacterial effects were observed after 10 min. After 1 h, the antibacterial effects of the pastes were clearly hampered by the necrotic tissue, since the number of bacterial cells was far larger than the controls with no tissue and comparable to the positive controls with saline and no medicament. However, after 1 day, all pastes succeeded in completely eliminating *E. faecalis* cells, except for CHCx, which caused an abrupt reduction

Table 1. Antimicrobial effects of medicaments in the presence of necrotic soft tissue. Tissue was contaminated with *Enterococcus faecalis* prior to testing.

	1 h		1 day		2 days	
	Control	Necrotic tissue	Control	Necrotic tissue	Control	Necrotic tissue
Saline	4.31×10^7	-	2.76×10^8	-	3×10^7	-
CHG	6.83×10^7	7.54×10^7	0	0	0	0
CHPG	8.18×10^7	8.23×10^7	0	0	0	0
CHCx	6.2×10^7	4.85×10^7	0	0	0	0
CxZO	6.9×10^7	3.58×10^7	0	7.1×10^5	0	7.1×10^4

CHG = calcium hydroxide/glycerin paste; CHPG = calcium hydroxide/camphorated paramonochlorophenol/glycerin paste; CHCx = calcium hydroxide/chlorhexidine gluconate paste; CxZO = chlorhexidine gluconate/zinc oxide paste.

Table 2. Antimicrobial effects of medicaments in the presence of necrotic soft tissue. Tissue was contaminated with *Candida albicans* prior to testing.

	1 h		1 day		2 days	
	Control	Necrotic tissue	Control	Necrotic tissue	Control	Necrotic tissue
Saline	4.7×10^6	-	1.19×10^5	-	1×10^4	-
CHG	1.88×10^6	1.14×10^6	0	3×10^2	0	0
CHPG	4.9×10^5	3.1×10^5	0	0	0	0
CHCx	4.1×10^5	1.7×10^5	0	0	0	0
CxZO	3.54×10^6	2.2×10^6	0	1.16×10^6	0	4.6×10^3

*For abbreviations see footnote to Table 1.

in the number of cells, but not total elimination (Table 3).

Experiment 3

When medicaments were maintained in serum for 9 days before the antimicrobial test, the qualitative results of the 1-day period for both *E. faecalis* and *C. albicans* revealed that only CHG and CHCx had a reduction in efficacy (Tables 4 and 5). No growth was observed for these 2 microorganisms when in contact with medicaments for 1 day without the influence of serum.

Table 3. Antimicrobial effects of medicaments against *Enterococcus faecalis* in the presence of necrotic soft tissue. Medicaments were incubated with necrotic tissue prior to testing.

	10 min		1 h		1 day	
	Control	Necrotic tissue	Control	Necrotic tissue	Control	Necrotic tissue
Saline	1.8×10^8	-	1.3×10^8	-	1.2×10^8	-
CHG	1.3×10^8	1.4×10^8	2.6×10^3	1.1×10^8	0	0
CHPG	1×10^8	1.2×10^8	4.2×10^4	1×10^8	0	0
CHCx	1.5×10^8	1.2×10^8	5.2×10^4	1×10^8	0	1.9×10^2
CxZO	1.1×10^8	1×10^8	4.9×10^4	1.1×10^8	0	0

*For abbreviations see footnote to Table 1.

Table 4. Antimicrobial effects of medicaments against *Enterococcus faecalis* in the presence of serum. Medicaments were incubated with serum prior to testing.

	30 min		1 h		1.5 h		1 day	
	Control	Serum	Control	Serum	Control	Serum	Control	Serum
Saline	+	+	+	+	+	+	+	+
CHG	+	+	+	+	+	+	-	+
CHPG	+	+	+	+	+	+	-	-
CHCx	+	+	+	+	+	+	-	+
CxZO	+	+	+	+	+	+	-	-

*For abbreviations see footnote to Table 1.

Table 5. Antimicrobial effects of medicaments against *Candida albicans* in the presence of serum. Medicaments were incubated with serum prior to testing.

	30 min		1 h		1.5 h		1 day	
	Control	Serum	Control	Serum	Control	Serum	Control	Serum
Saline	+	+	+	+	+	+	+	+
CHG	+	+	+	+	+	+	-	+
CHPG	+	+	+	+	+	+	-	-
CHCx	+	+	+	+	+	+	-	+
CxZO	+	+	+	+	+	+	-	-

*For abbreviations see footnote to Table 1.

DISCUSSION

Interappointment intracanal medication is mostly recommended to supplement the antimicrobial effects of chemomechanical preparation by acting on areas not reached by instruments and irrigants including dentinal tubules, isthmuses and ramifications. For intracanal medicaments to exert their antimicrobial effects in those regions, they have to diffuse into them, not to be significantly influenced by organic and inorganic tissue components and then reach a concentration that is high enough to kill or be inhibitory to microorganisms residing in those locations.

Studies have demonstrated that several irrigants and medicaments can be somewhat influenced by organic and/or inorganic tissue components. For instance, CH has been shown to be totally inactivated by the presence of dentin, hydroxyapatite or bovine serum albumin (6,10). Cx effects can be slowed down by dentin and strongly inhibited by bovine serum albumin and heat-killed bacteria (6,10,11). Iodine potassium iodide can be totally inhibited by dentin, dentin matrix, and heat-killed microbial cells (6,10,11).

The microbial species selected for use in this study have been commonly detected in cases of persistent/secondary endodontic infections (8,9). Both of them can be easily grown under aerobic conditions and have shown resistance to substances used in endodontic treatment, including CH (12). Indeed, such a resistance to CH has been one factor that has prompted researchers to propose association of CH with other substances, such as CPMC and Cx, so as to increase the spectrum of antimicrobial activity to include these resistant species (13-15).

In the first experiment, the necrotic tissue was not mixed with the medicaments in an attempt to simulate the clinical condition where the medicament has to diffuse to exert its effects. Under this condition, CxZO was significantly affected in its efficacy against the 2 test species. To a lesser extent, CH in an inert vehicle was also affected against *C. albicans*. When the medicaments were mixed with necrotic tissue before testing (Experiment 2), all medicaments were somewhat affected after 1 h of contact with bacteria. However, after one day, only CH in Cx was significantly affected.

Our overall results indicated that the necrotic tissue slowed down the antimicrobial effects of all medicaments. These inhibitory effects are likely to be related to the tissue content of proteins, although byproducts of tissue degeneration may also have had some negative impact on the antimicrobial activity. However, these effects were time-dependent. It remains to be established if, in the clinical setting, the time medicaments are usually applied (7 days) is sufficient to exceed these inhibitory effects to the point of providing predictable disinfection in areas distant from the main canal.

Among the medicaments tested in this study, CHPG seemed to be the least affected by either necrotic tissue or serum. This combination has already been demonstrated to exhibit pronounced antibacterial activity against several candidate endodontic pathogens (13,14,16). Although CPMC exhibits high toxicity when used alone, satisfactory biocompatibility results have been observed for its association with CH in animal studies (17,18). Clinical studies evaluating the incidence of postoperative pain (19), antibacterial activity (3) and treatment outcome (20) have demonstrated excellent results when using an antibacterial protocol for treatment that includes a 7-day interappointment medication with CHPG.

In conclusion, the antibacterial activity of all intracanal medicaments tested in the present study can be slowed down in the presence of necrotic soft tissue. CH pastes using glycerin or chlorhexidine as vehicles were significantly affected by serum. Of the tested medicaments, the least affected by serum and necrotic soft tissue was the CH/camphorated paramonochlorophenol/glycerin paste.

RESUMO

O objetivo deste estudo foi avaliar a influência do soro e de tecido mole necrosado na atividade antimicrobiana de medicamentos intra-canais. Os medicamentos testados foram pastas de hidróxido de cálcio/glicerina, hidróxido de cálcio/clorexidina, hidróxido de cálcio/paramonoclorofenol canforado/glicerina e clorexidina/óxido de zinco. A sobrevivência de *Enterococcus faecalis* e *Candida albicans* expostos aos medicamentos na presença ou ausência de soro ou tecido necrosado foi monitorada em três experimentos *in vitro* nos quais amostras para cultura foram avaliadas em diferentes períodos de tempo. No geral, os resultados demonstraram que a atividade antimicrobiana de todos os medicamentos testados foi retardada na presença de soro ou de tecido necrosado. As pastas de hidróxido de cálcio em glicerina ou clorexidina foram significativamente afetadas pelo soro. Dos medicamentos testados, o menos afetado foi a pasta de hidróxido de cálcio/paramonoclorofenol canforado/glicerina.

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