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ORIGINAL ARTICLE

Effects of Osmotic Stress and Sodium Hypochlorite on Endodontic Microbiota: An *In-Vitro* Study

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ABSTRACT

Objective: To assess the effect of osmotic stress on various bacteria in a planktonic milieu and the effect of exposure to sodium hypochlorite (NaOCl) on the microbial cells previously subjected to osmotic stress.

Methods: *Enterococcus faecalis*, *Streptococcus sanguinis, Fusobacterium nucleatum, Porphyromonas gingivalis* and *Prevotella intermedia* were suspended as follows: Iso-osmotic group 0.9% NaCl; Hypo-osmotic group "ultrapure water"; Hyper-osmotic group 9% NaCl solution for 120 hours before exposure to 0.0001% NaOCl for 10 minutes. Quantitative analyses of viable cells were performed at 0 and 120 hours and after exposure to NaOCl to obtain colony forming units (CFU/mL). A linear mixed-effects model was used to find the association between mean CFU/mL (logarithmic transformation) and the interaction of solution Group and Time (P<0.001).

Results: *F. nucleatum, P. gingivalis* and *P. intermedia* did not survive after 24 hours in any of the solutions and were excluded from further testing. For *S. sanguinis* there were significant differences at each time interval, when holding solution group constant. After 120 hours, the Hyper-osmotic group presented with the highest CFU/mL and was significantly different to the Iso-osmotic group (P<0.001). For *E. Faecalis*, there was a significant difference for each pairwise comparison of time (P<0.001) in mean CFU/mL between 0 hours and 120 hours for the Iso-osmotic and Hyper-osmotic groups. At 120 hours, no significant differences were found between the three groups. Significant differences were also found between 0 hours and Post-NaOCl administration, and between 120 hours and Post-NaOCl administration for all three groups (P<0.001). Exposure to NaOCl after hypo-osmotic stress was associated with significantly less CFU/mL for *S. sanguinis* compared to hyperosmosis and iso-osmosis (P<0.001) and for *E. Faecalis* only compared to hyperosmosis (P<0.001).

Conclusion: *S. sanguinis* and *E. faecalis* were able to withstand osmotic stress for 120 hours. Hypo-osmotic stress before contact with NaOCl was associated with lower viable bacterial numbers, when compared to the other media for the above species. Hyper-osmotic stress was associated with higher viable bacterial numbers after NaOCl exposure for *E. faecalis*.

Keywords: *Enterococcus faecalis*, osmosis, osmotic shock, sodium hypochlorite, stress response

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HIGHLIGHTS

- *Streptococcus sanguinis* and *Enterococcus faecalis* planktonic cells can survive in different osmotic stress conditions.
- Hypo-osmotic stress *S. sanguinis* and *E. faecalis* in the planktonic form are associated with increased susceptibility to sodium hypochlorite.
- Hyper-osmotic stress is associated with increased survival following exposure to sodium hypochlorite for *E. faecalis* in planktonic form.

INTRODUCTION

Microorganisms in the root canal system, particularly in post-treatment endodontic disease, subsist in a hostile milieu. Environmental stresses are external factors that harm the physiological welfare of cells, potentially resulting in a reduction in growth rate, or cell lysis (1). These stresses include extremes of temperature, pH, osmotic pressure, depletion of nutrients and the presence of toxic or inhibitory substances (2). Changes in osmotic pressure, in particular, is one of the most significant physical parameters with which bacteria must contend (3). Osmotic stress can vary from being hyper-osmotic, with an increase in external osmolality, to hypo-osmotic, with a decrease in external osmolality (4).

The technical procedures associated with root canal treatment lead to further environmental stresses which include osmotic shock (5). Commercially available sodium hypochlorite (NaOCl) prepa-

rations contain sodium chloride (NaCl) (6), and present with widely variable osmolarity values (7). Therefore, alongside the diffusion of active chlorine to the target areas (7), the dispersion of solutes originally contained in irrigant solutions should be expected to cause hyper-osmotic stress of unpredictable severity to the microorganisms present in the root canal system, with both mechanisms of action potentially working synergistically. Microorganisms that remain viable following post-treatment endodontic disease must also be able to adapt to survive the multiple stresses related to the root canal treatment. Though with limited clinical translation in endodontics, hypo-osmotic stress has been suggested to promote leakiness of cellular membranes (4), which may subsequently favour penetration of biocidal agents into the bacterial cytoplasm (5).

Planktonic microorganisms play a significant role in diseases and are a prerequisite for biofilm formation. In addition, microorganisms detach from mature biofilms with this being a continuous process associated with spread and colonisation of other surfaces (8). Therefore, in endodontics, planktonic bacteria are responsible for the initial colonisation, and eventual recolonisation of the treated canal after clinical procedures are completed. In the latter situation, planktonic microorganisms present outside the main root canal space are a likely source of reinfection. In the oral cavity Streptococci species have a crucial role as early colonisers, being capable of interacting with the conditioning film present on the tooth surface (9). After adherence by the primary colonisers, secondary colonisers attach leading towards further biofilm development (9). Finally, it should be noted that symptoms and exacerbations are caused by planktonic bacteria, whereas the presence of a biofilm component of the root canal infection guarantees persistence of such infection (10).

Considering the paucity of information regarding the effect of osmotic stress on planktonic bacteria commonly associated with root canal infection, the aim of the current study was twofold: i) to assess the effect of environmental osmotic stress on various bacterial species associated with root canal infection in a planktonic milieu, ii) to assess the effect of exposure to NaOCl on the microbiota previously subjected to osmotic stress. The null-hypothesis tested was that are no differences in the number of viable bacteria at the different time points, following exposure to osmotic stress and contact with NaOCl, when testing various species associated to root canal infection.

MATERIALS AND METHODS

Bacterial isolates

Bacterial species selected for this experiment were: *Enterococcus faecalis* (V583 ATCC 700802), *Streptococcus sanguinis* (NCTC 7862) and Fusobacterium nucleatum (ATCC 25586), *Porphyromonas gingivalis* (W50) and *Prevotella intermedia* (ATCC 25611). All strains were stored as frozen stock cultures in 40% v/v glycerol at –80°C.

Bacterial cultivation

Before use, *E. faecalis* and *S. sanguinis* were cultured aerobically and maintained on Todd Hewitt Agar (Oxoid, Victoria, Australia) and Tryptone Soya Agar (Oxoid) for 24 hours at 37°C respectively. *F. nucleatum*, *P. gingivalis* and *P. intermedia* were grown on Anaerobic Blood Agar (Oxoid) under an atmosphere of 5% CO_2, 5% H_2 and 90% N_2 for 72 hours at 37°C throughout the assays.

For all experiments using planktonic cultures, Todd Hewitt Broth and Tryptone Soya Broth (TSB) was the growth medium for *E. faecalis* and *S. sanguinis* respectively. Heart Infusion Broth supplemented with Vitamin K (1 µg/mL) and Hemin (5 µg/mL) was used as the growth medium for *F. nucleatum*, *P. gingivalis* and *P. intermedia*. The purity of cultures was confirmed routinely as follows: *E. faecalis*: Gram staining, colony morphology and growth on bile aesculin agar (Oxoid); *S. sanguinis*: growth on Mitis Salivarius agar (Difco, East Rutherford, NJ, USA); *F. nucleatum*, *P. gingivalis* and *P. intermedia* growth on Anaerobic blood agar plates containing nalidixic acid and vancomycin (Oxoid).

Effect of starvation and osmotic stress

Individual colonies were grown to late-log phase and cells harvested by centrifugation (10.000X g for 15 minutes, 4°C) and washed twice in 0.9% w/v NaCl (UNIVAR, Seattle, USA) and re-suspended in the following conditions: Iso-osmotic group 0.9%, NaCl; Hyper-osmotic, group Milli-Q "ultrapure water" (Merck Millipore); Hyperosmotic group 9% NaCl solution. The pH of the solutions was 7. All cultures were adjusted to an optical density of 1.000±0.050 at 600 nm using a Nanodrop 2000 spectrophotometer (Thermofisher Scientific, Coresby, Australia). At 120 hours, serial dilutions were performed on cell cultures followed by plating and incubation at 37°C for 24 hours. Quantitative analysis of viable cells was performed by serial dilution to give colony forming units (CFU/mL).

Conductivity measurements - *E. faecalis* and *S. sanguinis*

The aliquots of the microbe and relevant solution were kept at room temperature. At time intervals of 24, 48, 72, 96 and 120 hours, aliquots were removed, centrifuged (10,000 X g, 15 minutes, 4°C) and the conductivity (LAQUAtwin, HORIBA Scientific, Kyoto, Japan) of the decanted supernatant was recorded at each time interval as a measurement of osmolarity. At the completion of the experiment, the purity of cultures was confirmed as described previously.

Exposure of osmotically stressed bacterial strains to sodium hypochlorite

All bacterial strains that had been subjected to the test conditions were then subjected to a freshly prepared solution of 0.0001% NaOCl for 10 minutes, which was added to the original solution. This concentration was decided based on previous comparable studies (11, 12). Following the exposure for 10 minutes, 100 µL 5% sodium thiosulphate ($Na_2S_2O_3$) (Sigma Aldrich, North Ryde, Australia) was added as a neutralising agent. Serial dilutions were then carried out and quantitative analysis through enumeration of colonies (CFU/mL) and conductivity measurements were again completed. All combinations of the test solution and bacterial strain were repeated with triplicate plating, including T=0.

Statistical analysis

The statistical analysis was performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). A linear mixed-effects model

was used to find the association between mean CFU/mL (logarithmic transformation) and the interaction of solution Group and Time. The quantitative variables were summarised with mean, confidence levels and standard deviations where applicable. A P-value<0.001 was considered statistically significant.

RESULTS

Based on our preliminary studies, a NaOCl concentration of 0.0001% with a contact time of 10 minutes was found to be sub-lethal (data not shown). Similarly, a 5% concentration of $Na_2S_2O_3$ used as the neutralising agent was confirmed to be non-toxic to the bacteria used in the study (data not shown).

S. sanguinis

The baseline (0 hours) mean CFU/mL was not significantly different amongst the three groups (P≥0.001). There were significant differences at each time interval, when holding solution group constant (P<0.001). After 120 hours, the Hyper-osmotic group presented with the highest CFU/mL and was significantly different to the Iso-osmotic group (P<0.001). Following NaOCl exposure, the Hypo-osmotic group presented with the lowest CFU/mL and was significantly different to the Iso-osmotic and Hyper-osmotic groups (P<0.001). CFU/mL and standard deviations for each group at time intervals of 0 hours, 120 hours and Post-NaOCl are shown in Table 1.

E. faecalis

The CFU/mL at T=0 were not significantly different amongst the three groups (P≥0.001). For each pairwise comparison of time, holding solution group constant, there were significant differences (P<0.001) in mean CFU/mL between 0 hours and 120 hours for the Iso-osmotic and Hyper-osmotic groups. Significant differences (P<0.001) were also found between 0 hours and Post-NaOCl administration, and between 120 hours and Post-NaOCl administration for all three groups. At 120 hours, no significant differences were found between the three groups. Following NaOCl exposure, the Hypo-osmotic group presented with the lowest mean CFU/mL(P<0.001) compared to the Hyper-osmotic group. There was also a significant difference between the Iso-osmotic and Hyper-osmotic group (P<0.001), with the Iso-osmotic group displaying a greater mean CFU/mL reduction. Table 2 shows the mean CFU/mL and standard deviations for each Group at time intervals of 0 hours, 120 hours and Post-NaOCl for *E. faecalis*.

F. nucleatum, *P. gingivalis* and *P. intermedia*

F. nucleatum, *P. gingivalis* and *P. intermedia* did not survive after 24 hours in any of the solutions, therefore these were excluded from further experiments.

Conductivity measurements

Conductivity values for *S. sanguinis* and *E. faecalis* were found to be similar. Minimal changes occurred for the Hyper-osmotic and Iso-osmotic groups at different time points. In the Hypo-osmotic groups, after an initial increase, conductivity values reduced over time, in particular for *S. sanguinis*, with the addition of NaOCl leading to a notable increase in values. The mean conductivity values for *S. sanguinis* and *E. faecalis* are shown in Figures 1 and 2, respectively.

DISCUSSION

S. sanguinis and *E. faecalis* were able to withstand the different osmolarity stresses for 120 hours. It should be noted that the microbial cells may have also been exposed to starvation stress, as the only solute present was NaCl. Exposure to NaOCl after hypo-osmotic stress was associated with significantly less CFU/mL for *S. sanguinis* overall and *E. faecalis* when compared with hyper-osmotic stress. *E. faecalis* presented with higher viable bacteria numbers following exposure to NaOCl after suspension in the Hyper-osmotic solution. Therefore, the null-hypothesis was partially rejected. Furthermore, high conductivity values were found for both isolates after exposure to NaOCl in the hypo-osmotic solution.

The present study assessed the effect of exposure to osmotic stress followed by NaOCl on species commonly associated with primary and secondary root canal infections. Resistance to osmotic stresses may be one of several capabilities allowing

TABLE 1. Mean and standard deviations of CFU/mL for *S. sanguinis* (NCTC 7862), (n=6)

Time	Hyper-osmotic		Hypo-osmotic		Iso-osmotic	
	Mean	SD	Mean	SD	Mean	SD
0 hours	$5.69x10^{9a}$	$5.45x10^{8}$	$5.39x10^{9a}$	$7.86x10^{8}$	$6.65x10^{9a}$	$2.90x10^{8}$
120 hours	$2.68x10^{9b}$	$5.69x10^{8}$	$2.01x10^{9b, c}$	$2.90x10^{8}$	$1.54x10^{9c}$	$1.63x10^{8}$
Post-NaOCl	$1.27x10^{8d}$	$1.07x10^{7}$	$7.63x10^{6e}$	$6.21x10^{5}$	$1.34x10^{8d}$	$1.36x10^{7}$

NaOCl: Sodium hypochlorite. Different superscript letters represent significant differences based on a linear mixed-effects model. Statistically significant at P<0.001

TABLE 2. Mean and standard deviations of CFU/mL for *E. faecalis* (V583 ATCC 700802), (n=6)

Time	Hyper-osmotic		Hypo-osmotic		Iso-osmotic	
	Mean	SD	Mean	SD	Mean	SD
0 hours	$1.37x10^{11a}$	$3.06x10^{10}$	$1.25x10^{11a}$	$8.66x10^{10}$	$2.07x10^{11a}$	$1.67x10^{10}$
120 hours	$1.60x10^{10b}$	$7.81x10^{9}$	$3.75x10^{10b}$	$1.02x10^{9}$	$1.47x10^{10b}$	$5.45x10^{9}$
Post-NaOCl	$3.10x10^{8d}$	$5.41x10^{7}$	$2.70x10^{7c, e}$	$4.09x10^{6}$	$4.80x10^{7e}$	$1.19x10^{6}$

NaOCl: Sodium hypochlorite. Different superscript letters represent significant differences based on a linear mixed-effects model. Statistically significant at P<0.001

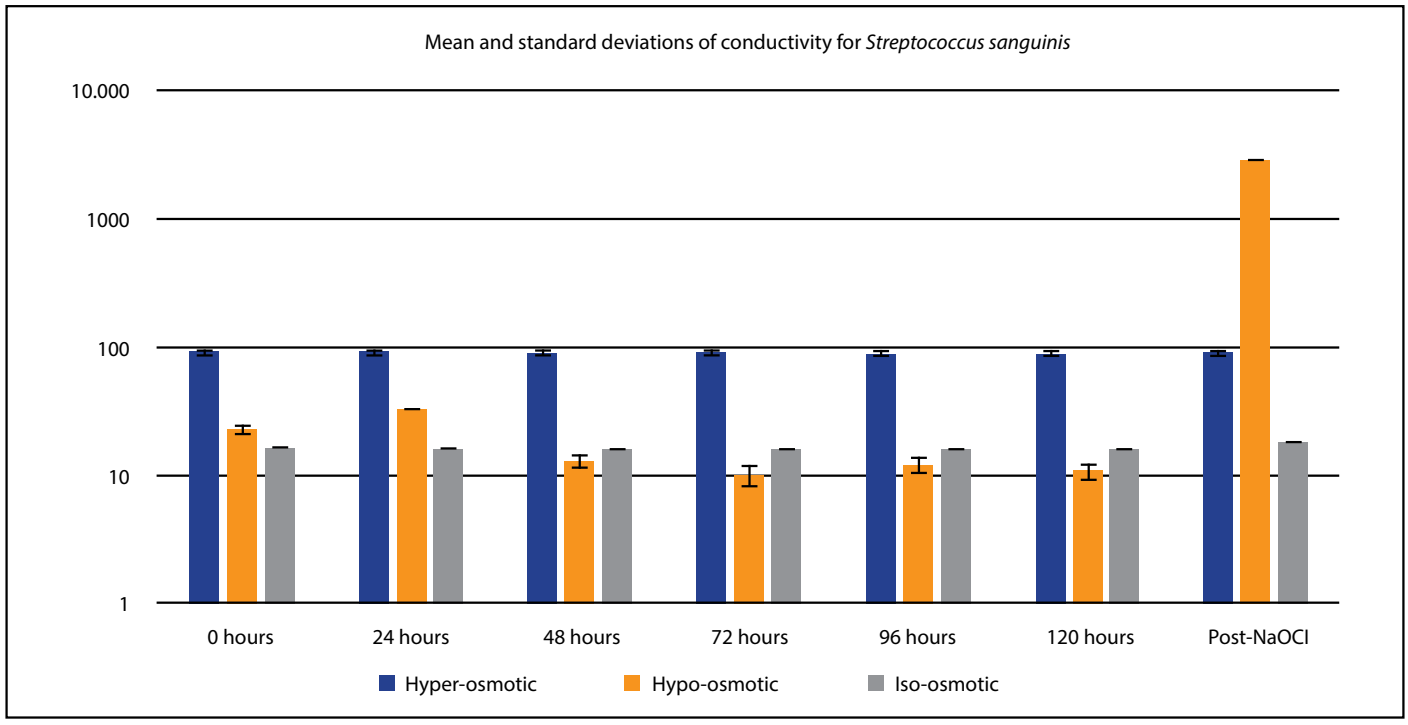

Figure 1. Mean and standard deviations of conductivity for *S. sanguinis* (NCTC 7862)
NaOCl: Sodium hypochlorite

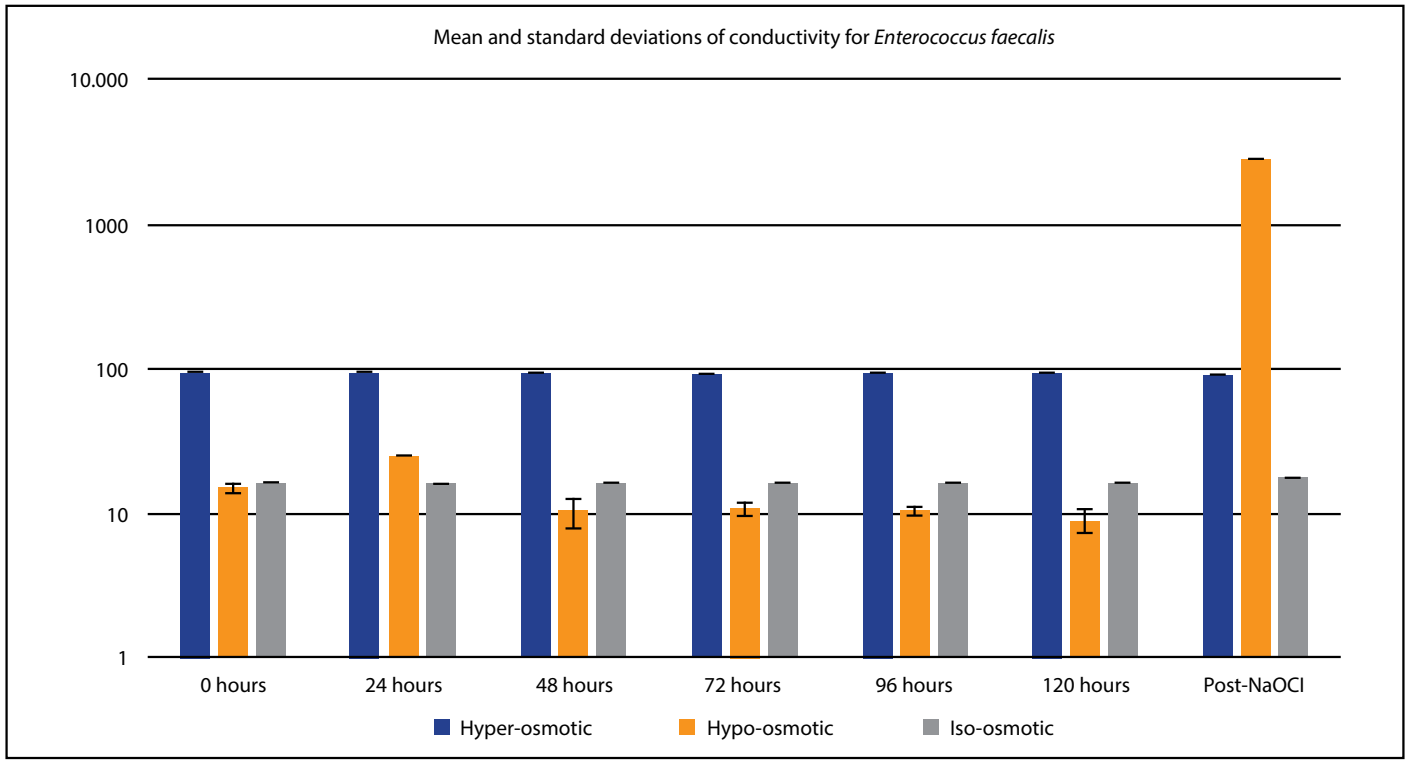

Figure 2. Mean and standard deviations of conductivity for *E. faecalis* (V583 ATCC 700802)
NaOCl: Sodium hypochlorite

S. sanguinis and *E. faecalis* to survive the procedures related to root canal treatment and the subsequent post-treatment environment. Furthermore, *E. faecalis* has also the ability to survive alkaline stress (13), and NaOCl is both hypertonic (6, 7) and alkaline (14). These species are part of the microbiota commonly associated with failed root canal treatment (15). Conversely, the test species considered representative of the polymicrobial intraradicular flora in untreated teeth (i.e. *F. nucleatum*, *P. gingivalis* and *P. intermedia*) (15) were unable to survive for 24 hours in any of the solutions. The presence of specific cell wall polysaccharides (16) and their ability for remodelling the cell envelope may confer resistance to osmotic stress to *S. sangui-*

nis and *E. faecalis* (17, 18), though this may depend on several factors.

The present study tested different isolates in planktonic conditions, however, the eradication of intraradicular infection is considered challenging because biofilm renders the microbiota more resistant to treatment procedures (19). Conversely, it should be noted that there is evidence showing no difference between planktonic and biofilm forms, and that it is in the stationary phase when cells display maximum resistance (20). In fact, planktonic cells present with alternative survival mechanisms (13). It should be noted that endodontic studies commonly compare biofilms with planktonic cultures before reaching the stationary phase (13, 21), thus more sensitive to biocidal agents (11).

It is also probable that the physiological state of the bacterial cells in this starvation phase would best mimic the physiological state of the cells in the root canal, particularly in post-treatment disease (11). Considering that all microbial growth forms within root canals have been observed including sessile biofilms, aggregates and planktonic forms, and the physiological state of the microbe influences resistance (11), a planktonic model has been proposed based on simplicity, accuracy, and reproducibility (22). It should also be noted that planktonic killing tests allow the testing of agents in direct contact with microorganisms and free of potential confounding factors (23).

The conductivity of an electrolyte solution is used to evaluate its ionic content (24), and was used to validate the osmolarity values of the cultures tested. In the present study, largely, the Hypo-osmotic groups presented with decreasing conductivity levels until exposure to NaOCl, which resulted in a rapid increase in conductivity. The reduction of conductivity may be associated with the uptake of ions (i.e. NaCl) from the medium (25), though with hypo-osmotic shock, it is purported that there would be an influx of water into the cells with a subsequent build-up of turgor pressure (4). The increased turgor in a hypo-osmotic environment may lead to the bursting of bacterial cells (26), and a subsequent surge in conductivity. Remarkably, the bursting of bacterial cells will cause a hyper-osmotic milieu locally. Uptake or release of osmotically-active solvents is a strategy often used by microorganisms exposed to osmotic stress.

The literature assessing the role of osmotic stress in endodontology is scarce and focusses solely on hyper-osmotic stress (1, 5, 27, 28). In 4-day old mixed biofilms of *E. faecalis* and Pseudomonas aeruginosa, a Hyper-osmotic solution (brain heart infusion broth with 6 mol L-1 NaCl) had a highly significant negative effect on biofilm viability with a linear trend in time, after 72 and 168 hours. Furthermore, a reduction of the biofilm mass (i.e. extracellular polysaccharides) after 4 hours occurred in 1-day old biofilms (27). *E. faecalis* was more resistant to the stress than P. aeruginosa.. A further study from the same group found that NaCl (1 M) or potassium sorbate (KS) (1 or 3.9 M) alone were unable to eradicate 48 h old *E. faecalis* biofilms, though their association (3M NaCl+1 M KS) can achieve this aim (1). Finally, a solution containing NaCl and KS detaches biofilm cells whilst having an antimicrobial effect (28). It should be noted that in these studies, immature biofilms were assessed which are more sensitive to agents compared to mature biofilms (29), and possibly cells in the stationary state (20).

The present study assessed hypo-osmosis on bacteria associated with root canal infection and the clinical application of this effect requires further understanding. Dilution of cells into media of low osmolality may, however, confer a generalised leakiness to the membranes (5, 30). Theoretically, this may promote the penetration of active components into the bacterial cells and influence their viability (5). Future disinfection strategies should consider the use of hypo-osmotic stress before the use of intra-canal biocidal agents, for example, NaOCl.

The ability of *E. faecalis* to survive hyperosmosis is not novel. Seminal investigations utilised its specific ability to grow in the presence of 6.5% NaCl, incorporated into either a broth or an agar medium for the presumptive identification of the enterococcal group D organisms. The salt-tolerance tests, along with the bile-esculin test, helps distinguish the Enterococcus species from the group D Streptococci, Streptococcus bovis and Streptococcus lactis (31). The finding that Enterococcus species can tolerate high salt conditions is not surprising considering that they are normal commensals in complex hyper-osmotic environments such as the gastrointestinal tract, which contains bile salts, amongst others (32, 33). In fact, *E. faecalis* can respond to frequently occurring environmental fluctuation as these may occur frequently in the course of life of enteric bacteria (32, 33).

Exposure to the hyper-osmotic stress before NaOCl was associated with higher viable bacteria numbers for *E. faecalis*, when compared to the other media. This may be related to cross-resistance after hyper-osmotic shock, that in addition to other capabilities, including cross-protection associated with other various stress responses (32, 33), and the ability to act as a recipient of genetic material present in endodontic biofilms (34), allow *E. faecalis* to survive extreme challenges. Therefore, from the clinical standpoint, the importance of a robust chemo-mechanical instrumentation, in order to eliminate intra-radicular infection and to prevent cross-protection associated with the different sub-lethal stresses, should be reiterated. Cross-protection may be of particular relevance for those cells associated with exacerbations of chronic diseases, such as chronic apical periodontitis, which is caused by root canal infection (15).

The present study has limitations. This study examined selected microorganisms only in the planktonic form. In addition, root canal infections commonly involve multispecies infections. Further studies assessing the effects of osmotic stress in relevant root canal multispecies biofilm models are required.

CONCLUSION

S. sanguinis and *E. faecalis* in the planktonic form were able to withstand osmotic stress for 120 hours, whereas exposure to hypo-osmotic stress before contact with NaOCl was associated with lower viable bacteria numbers, when compared to exposure to an iso-osmotic or a hyper-osmotic medium. *E. faecalis* previously exposed to the hyper-osmotic stress had higher bacterial numbers following exposure to NaOCl, compared

to the other media. *F. nucleatum*, *P. gingivalis* and *P. intermedia* were unable to survive similar conditions.

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