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Niazi, SA, Clark, D, Do, T orcid.org/0000-0002-5668-2181 et al. (4 more authors) (2014)
The effectiveness of enzymic irrigation in removing a nutrient-stressed endodontic multispecies biofilm. *International Endodontic Journal*, 47 (8). pp. 756-768. ISSN 1365-2591

<https://doi.org/10.1111/iej.12214>

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The effectiveness of enzymic irrigation in removing a nutrient-stressed endodontic multispecies biofilm

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Abstract

Niazi SA, Clark D, Do T, Gilbert SC, Foschi F, Mannocci F, Beighton D. The effectiveness of enzymic irrigation in removing a nutrient-stressed endodontic multispecies biofilm. *International Endodontic Journal*.

Aim To establish a nutrient-stressed multispecies model biofilm and investigate the dynamics of biofilm killing and disruption by 1% trypsin and 1% proteinase K with or without ultrasonic activation.

Methodology Nutrient-stressed biofilms (*Propionibacterium acnes*, *Staphylococcus epidermidis*, *Actinomyces radicidentis*, *Streptococcus mitis* and *Enterococcus faecalis* OMGS 3202) were grown on hydroxyapatite discs and in prepared root canals of single-rooted teeth in modified fluid universal medium. The treatment groups included trypsin, proteinase K, 0.2% chlorhexidine gluconate and 1% sodium hypochlorite (NaOCl) (with and without ultrasonics). NaOCl and chlorhexidine were the positive controls and untreated group, and sterile saline was the negative control. The biofilms were investigated using confocal laser scanning microscopy (CLSM) with live/dead staining and quantitative microbial culture.

Results Nutrient stress in the multispecies biofilm was apparent as the medium pH became alkaline, glucose was absent, and serum proteins were degraded in the supernatant. The CLSM showed the percentage reduction in viable bacteria at the biofilm surface level due to nutrient starvation. On the disc model, trypsin and proteinase K were effective in killing bacteria; their aerobic viable counts were significantly lower ($P < 0.01$) than the negative control and chlorhexidine. NaOCl was the most effective agent ($P < 0.001$). In the tooth model, when compared to saline, trypsin with ultrasonics caused significant killing both aerobically and anaerobically ($P < 0.05$). Chlorhexidine (1.46 ± 0.42), trypsin (3.56 ± 1.18) and proteinase K (4.2 ± 1.01) with ultrasonics were significantly effective ($P < 0.05$) in reducing the substratum coverage as compared to saline with ultrasonics ($12\% \pm 4.9$).

Conclusion Trypsin with ultrasonic activation has a biofilm killing and disrupting potential.

Keywords: stressed biofilm model.

Received 24 February 2012; accepted 13 November 2013

Introduction

The main objective of root canal treatment is the elimination of bacteria from the root canal system by chemomechanical means (Byström & Sundqvist 1981, Dalton *et al.* 1998). Root canal irrigants having tissue lytic and microbicidal activity facilitate the removal of unwanted organic and inorganic material (Byström & Sundqvist 1983, Sjögren *et al.* 1997, Shuping *et al.* 2000, Siqueira *et al.* 2002).

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Irrigants act by flushing loose debris and lubricating the dentinal walls (Siqueira *et al.* 2002). Sodium hypochlorite (1–5.25%) and chlorhexidine (0.2–2%) solutions are the most commonly used irrigants (Zehnder 2006, Giardino *et al.* 2007, Siqueira *et al.* 2007) and their effects can be enhanced by their use in combination with ultrasonic cleaning (Weller *et al.* 1980, Lee *et al.* 2004, van der Sluis *et al.* 2006). To exert their antimicrobial effect, irrigants should be able to disrupt the biofilm matrix, because the complex anatomy of the root canal system with lateral canals, isthmuses and apical deltas provides the bacterial biofilm with niches to escape these antimicrobial irrigants.

Proteinase K and trypsin are proteolytic enzymes, which have been used in studies to degrade the extracellular matrix leading to dissolution of staphylococcal and *Gardnerella vaginalis* biofilms (Chaignon *et al.* 2007, Patterson *et al.* 2007). Given their biofilm disruption potential, these proteases may have a possible role as advanced endodontic irrigants either as an alternative or as an adjunct to NaOCl and chlorhexidine. However, these agents need to be assessed initially in relevant biofilm models that simulate the *in vivo* environment as closely as possible. A variety of biofilm models have been used to test numerous endodontic irrigants (Hubble *et al.* 2003, Gulabivala *et al.* 2004, Hems *et al.* 2005, Kishen *et al.* 2006, Kowalski *et al.* 2006, Sena *et al.* 2006, Shen *et al.* 2009, 2010, Pappen *et al.* 2010). However, all models are at best approximations, and these models may not be the most appropriate as biofilms within infected root canals are multispecies and are subject to nutritional stress due to a limitation in the nutrient supply (Siqueira 2002). Under nutritional stress, organisms alter their cell morphology and cell surface, with enhanced adherence (Bowden & Li 1997).

In this study, the development of a nutrient-stressed multispecies biofilm model is reported, using bacteria isolated from refractory endodontic infections (Niazi *et al.* 2010). The model was used to investigate the killing and disruption of biofilm by trypsin and proteinase K with or without ultrasonic irrigation.

Materials and methods

Development of a multispecies biofilm

Propionibacterium acnes, *Staphylococcus epidermidis*, *Actinomyces radicidentis* and *Streptococcus mitis*

recovered in a previous study as predominant taxa (Niazi *et al.* 2010) from the root canals of teeth with refractory endodontic infections were selected. In addition, *Enterococcus faecalis* strain OMGS 3202 reported to be present in refractory lesions (Dahlén *et al.* 2000) was also included.

To establish the biofilms, the strains were cultured anaerobically at 37 °C on Fastidious Anaerobe Agar (FAA, Lab M, Lancashire, UK) supplemented with 5% defibrinated horse blood. Individual starter cultures of each species were established in filter-sterilized modified fluid universal medium (mFUM) (Gmur & Guggenheim 1983) and incubated anaerobically at 37 °C for 3 h. The absorbance was adjusted with fresh mFUM to 0.5 at 540 nm to obtain 10^7 cells mL⁻¹. (Labsystems iEMS Reader MF, Basingstoke, UK). Hydroxyapatite discs (9 mm diameter, Clarkson Chromatography Products Inc., South Williamsport, PA, USA) were autoclaved at 121 °C for 15 min, placed in 1 mL of mFUM and pre-reduced in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide). The hydroxyapatite discs were seeded with 400 µL (4×10^6 cells) of each of the five starter cultures. The discs were divided into three groups ($n = 5$ discs group⁻¹) and named as T0, T1 and T2. In the T0 group, the biofilms were grown anaerobically with regular medium change after every 24 h for the first 7 days. T1 and T2 biofilms were further grown in the same medium without changing the medium for an additional 7 and 14 days, respectively. The medium pH (Corning pH meter 240, Sigma-Aldrich, Dorset, UK) and glucose concentration (Glucose HK kit, Sigma-Aldrich, Dorset, UK) were determined, and protein profiles were examined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli method (Laemmli 1970).

To enumerate the numbers of bacteria in the biofilms, half of the biofilm on each disc was removed using a sterile scalpel blade (Swann-Morton, Sheffield, UK) under magnification and dispersed into 1 mL of BHI (Brain-Heart infusion Broth, Lab M, Lancashire, UK), by vortexing. After serial dilution in BHI, aliquots (100 µL) were plated onto duplicate FAA plates and incubated anaerobically for 7 days, and the colonies were counted. The remaining half of the biofilm was stained with live/dead Baclight bacterial viability kit (Invitrogen, Paisley, UK) and visualized under a Leica SP2 confocal laser scanning microscope. The biofilm structure was analysed using bioImage_L (Chavez de Paz 2009).

The effect of irrigants on the model biofilm grown on hydroxyapatite discs

The effects of four irrigants, 1% NaOCl, 0.2% w/v chlorhexidine gluconate, 100 µg mL⁻¹ trypsin (Sigma) in mFUM and 100 µg mL⁻¹ proteinase K in mFUM, and an mFUM only control, were determined on the stressed biofilm ($n = 5$ biofilms group⁻¹). The concentration of trypsin and proteinase K of 100 µg mL⁻¹ was based on the concentration of these proteases used in previous studies (Moscoso *et al.* 2006, Patterson *et al.* 2007). The irrigation was performed using a 27-gauge side-vented irrigating needle and 3-mL syringe (Monoject, Tyco Healthcare, Gosport, UK). The biofilm disc was placed in 9-cm Petri dishes (SLS, UK), and then, 6 mL of the test solution was gently poured on top of the disc, so that it flowed off and away from the biofilm at a constant rate over 2 min. To determine the effects of the irrigants, half of the biofilm was removed and processed as described above. They were plated onto duplicate FAA plates and incubated aerobically for 2 days and anaerobically for 7 days along with duplicate MacConkey agar plates incubated aerobically for 2 days. The relative proportions of each species were determined by identifying their individual colony morphology on selective medium (FAA aerobically, anaerobically and MacConkey) after respective treatments. The proportions of the untreated control group represent the relative proportions of each species before the treatment. The remaining portion of the biofilms were analysed by CLSM as described above.

Development of the stressed biofilm in single-rooted teeth

Fifty-five freshly extracted single-rooted teeth were obtained, after ethical approval was granted from the United Kingdom National Health Service (Research Ethics Committee Reference Number 10/H0804/056) and autoclaved individually at 121 °C for 15 min. To prepare the root canal, the crown of the tooth was removed, and the root length standardized to 15 mm. The canal was prepared as described previously to produce hemi-sectioned teeth, which could be reassembled for endodontic instrumentation (Bhuva *et al.* 2010). The modification to this model was the creation of 2 lateral canals; one in the apical 1/3 and another in the middle 1/3 of the root laterally on the side of the main canal of the chosen root half using an F1 ProTaper rotary file (Dentsply Maillefer). The

stressed biofilms were developed on the hemi-sectioned teeth using the above-mentioned protocol. After that, the root halves were reapproximated, the teeth were randomly allocated to eleven treatment groups, and their respective irrigation procedures were carried out (Table 1). For conventional syringe irrigation, a rubber stop was placed at a length of 14 mm from the tip of the irrigating needle. The needle was moved back and forth in the canal gently, ensuring that it did not bind in the canal. In case of ultrasonic irrigation, the power setting on the ultrasonic unit (Piezon Master 400; Electro Medical Systems, Nyon, Switzerland) was kept at one quarter of maximum for all of the roots. The size 15 ultrasonic file (Endosonore File, Dentsply Maillefer) was inserted into the canal as far as possible without obvious constraint of file oscillation.

After irrigation, the tooth was taken out of the silicon index, and the chosen root half with the two lateral canals was placed in the 9 cm Petri dish (SLS, Nottingham, UK). For microbial analysis, the samples were taken from the upper and lower half of the root canal, and the medial (close to the main root canal) and distal (close to the outer surface of the tooth) portion of middle 1/3 and the apical 1/3 of the artificially created lateral canal using sterile paper points (Protaper® Universal F3 Paper point Dentsply, Maillefer). Viable counts of all species were determined as described above, and the biofilm was also visualized using CLSM and analysed using *bioImage_L*.

Table 1 Treatments and procedures applied to the stressed biofilms developed in single-rooted teeth

Treatment Groups	Procedure
Control	Not exposed to any irrigant.
Chlorhexidine	Conventional syringe irrigation using
Trypsin	27-gauge side-venting irrigating needle
Proteinase K	and 3-mL syringe (Monoject, Tyco
NaOCl	Healthcare, Gosport, UK). Irrigation with
Saline	6 mL of solution was carried out at a
	constant rate for 2 min.
Chlorhexidine with ultrasonics	Ultrasonic irrigation with 4 mL solution using size 15 ultrasonic file (Obtura Spartan, Earth city, MO, USA) for 2 min.
Trypsin with ultrasonics	The irrigation time in this group was divided as follows: 30-s conventional syringe irrigation (1.5 mL), 20-s passive ultrasonic irrigation, 20-s syringe irrigation (1 mL), 20-s passive ultrasonic irrigation and then 30-s syringe irrigation (1.5 mL).
Proteinase K with ultrasonics	
NaOCl with ultrasonics	
Saline with ultrasonics	

Statistical analysis

The statistical analysis was performed in SPSSPC (Ver 16.0) using a general linear model for multivariate analysis of variance single factor: ANOVA was used to compare the quantitative viable counts between the treatment groups. *bioImage_L* (Chavez de Paz 2009) was used to compare the biovolume and biofilm substratum coverage in each treatment group by performing two-way analysis of variance (ANOVA).

Results

The characterization of the stressed *in vitro* multispecies biofilm

The mean number of bacteria (\pm SD) as \log_{10} (CFU per sample) in the biofilm at T0 was $6.89 (\pm 0.47)$ and $6.7 (\pm 0.31)$ at T1 and was reduced significantly ($P < 0.05$) to $5.89 (\pm 0.5)$ at T2. The pH value of the uninoculated mFUM was 7.19. At T0, it was as low as $5.58 (\pm 0.1)$, which significantly increased to $5.94 (\pm 0.13)$ at T1 ($P < 0.05$) and further increased to $6.13 (\pm 0.1)$ at T2 ($P = 0.05$); indicating extensive proteolysis. The glucose concentration of the uninoculated mFUM was 1.05 mg mL^{-1} but was reduced to 0.22 mg mL^{-1} at T0 and was $< 0.01 \text{ mg mL}^{-1}$ at both T1 and T2, respectively. The SDS-PAGE of

culture supernatants revealed loss of bands and the change in their mobility indicating degradation and loss of serum proteins at T1 and T2 (Fig. 1).

The CLSM analysis indicated that at T0, 99% of the biofilm bacteria at all levels throughout the biofilm were viable. At T1, the proportion of viable cells was significantly reduced, but at T2, the proportion of viable cells was $> 90\%$ at all levels (Fig. 2). The distribution at T1 and T2 was different to that observed at T0. It was decided to use cells at T1 for all the other parts of the study.

The effectiveness of irrigants on biofilms on hydroxyapatite discs

The mean number of bacteria (\pm SE) grown from the control discs, as \log_{10} (CFU per sample), was $8.07 (\pm 0.04)$ anaerobically, $7.86 (\pm 0.03)$ aerobically and $7.58 (\pm 0.04)$ on the MacConkey agar (Table 2). Chlorhexidine had no significant effect on the recovery of bacteria. Although trypsin and proteinase K also did not show any significant effect on the recovery of bacteria anaerobically or on MacConkey agar, they both significantly ($P < 0.05$) reduced the recovery aerobically. NaOCl was the most effective, significantly ($P < 0.001$) reducing the recovery on all media with mean values of < 10 CFU per sample (Table 2).

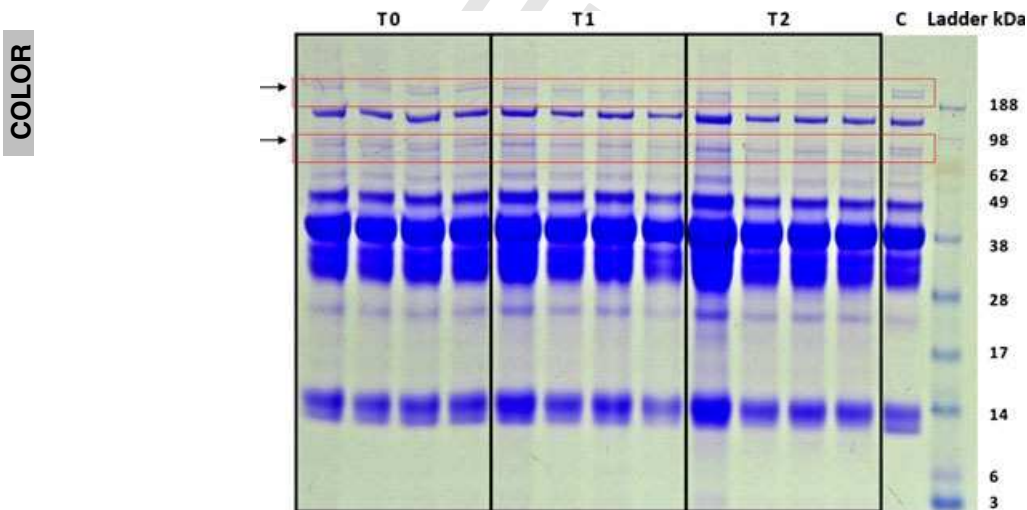


Figure 1 Comparison of total protein in the broth supernatant of three groups of biofilms T0, T1 and T2 separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Each group had four biofilms. T0 shows the protein separation of the broth supernatant for the biofilms grown for 7 days with regular medium change, whereas T1 and T2 represent the protein separation of broths of the biofilms left in the medium for another 7 and 14 days, respectively. Control (C) is the modified fluid universal medium.

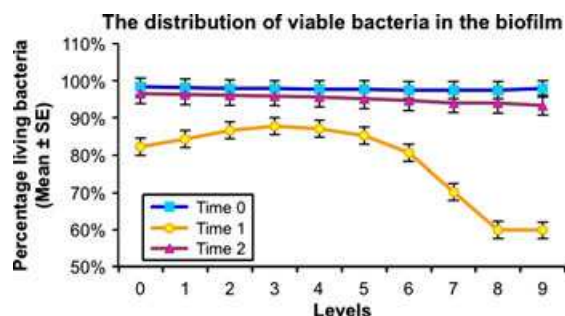


Figure 2 The distribution of the viable bacteria in all three groups of biofilm (group T0 is the biofilms grown for 7 days with regular medium change, whereas T1 and T2 represent the biofilms left in the medium for another 7 and 14 days, respectively). The *x*-axis shows the 9 levels of the *z* stack (level 0 is the deepest level of biofilm and level 9 is the most superficial level of the biofilm), and the *y*-axis gives the mean of the percentages of the living bacteria in all biofilms in each group.

According to the CLSM results, trypsin was most effective in bacterial killing amongst all four treatment groups as the mean per cent biovolumes for red (dead) populations were highest (55%) (Fig. 3a). The chlorhexidine group mean per cent biovolume of red (dead) populations was significantly less (21%) than that of both trypsin (55%) and proteinase K (37%) ($P < 0.05$). Compared with the control group, chlorhexidine exhibited no significant effect in the mean percentage biovolumes for green (75%) and red (21%) populations ($P > 0.05$). The mean per cent substratum coverage of the proteinase K group (74.1%) was significantly higher than the control group (54.3%) ($P < 0.05$), but there was no significant difference between the test groups (Fig. 3b).

Effects of irrigants on microbial killing on single-rooted teeth

The quantitative viable counts showed that the passive ultrasonic irrigation was significantly more effective in killing the biofilm bacteria as compared to the conventional syringe irrigation using the same irrigant ($P < 0.05$) (Table 3). NaOCl used with either conventional or ultrasonic irrigation proved to be the best irrigant with the viable counts significantly less than those of control, chlorhexidine, trypsin and proteinase K in all six sample sites ($P < 0.001$).

The viable counts for saline used with conventional or ultrasonics were significantly reduced ($P < 0.05$) as compared to the untreated control group. When

Table 2 Quantitative viable counts of the five disc model biofilm experimental groups

Treatment Groups	Microbial counts as \log_{10} per sample		
	Aerobic Mean \pm SE	Anaerobic Mean \pm SE	MacConkey Mean \pm SE
Untreated (Control)	7.86 (0.03) ***	8.07 (0.04) ***	7.58 (0.04) ***
Chlorhexidine	7.72 (0.05) ***	8.10 (0.07) ***	7.57 (0.10) ***
Trypsin	6.69 (0.13) *	8.00 (0.04) ***	7.40 (0.05) ***
Proteinase K	6.60 (0.02) *	7.90 (0.03) ***	7.69 (0.02) ***
Sodium hypochlorite	0.92 (4.232) **	0.50 (1.25) **	0.50 (1.25) **

*Values significantly less than control and chlorhexidine groups but greater than sodium hypochlorite ($P < 0.001$).

**Values significantly less than all other groups ($P < 0.001$).

***No significant difference in the values between the groups ($P > 0.05$).

compared to saline, proteinase K with conventional or ultrasonic irrigation and trypsin with conventional syringe irrigation caused no significant reduction in the viable counts ($P > 0.05$); however, trypsin with ultrasonics caused significant killing ($P < 0.05$) (Table 3).

In the case of chlorhexidine, the viable count values for chlorhexidine used with conventional or ultrasonic irrigation were significantly less than those of saline ($P < 0.05$). Moreover, chlorhexidine with ultrasonic irrigation was significantly more effective than both trypsin and proteinase K with conventional or ultrasonic irrigation ($P < 0.05$). Chlorhexidine with ultrasonic activation was more effective than conventional syringe irrigation in the medial portion of apical 1/3 of the artificially created lateral canal (Table 3).

Effects of irrigants on biofilm disruption on single-rooted teeth

The effects of the irrigants with or without ultrasonics on the tooth model are shown in Fig. 4(a,b). The substratum coverage of all the treatment groups was significantly less than the untreated control ($P < 0.001$) (Fig. 4b), and the majority of bacteria remaining after treatments were viable (Fig. 4a). This was in contrast to the effects of the irrigants on the substratum

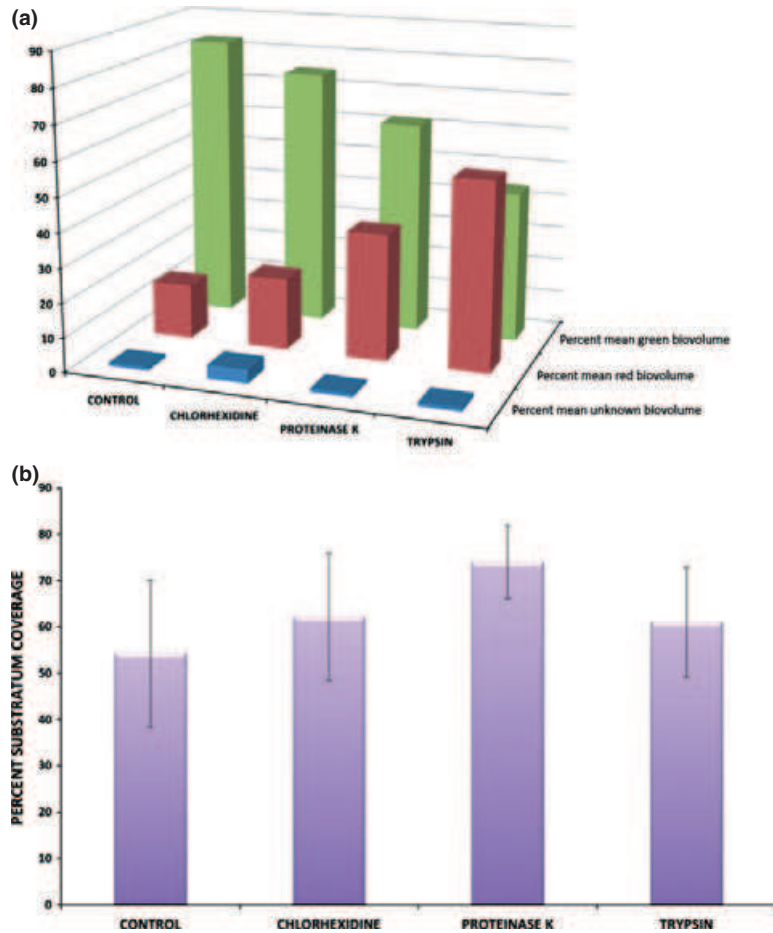


Figure 3 The effect of irrigant treatment on biofilms grown on hydroxyapatite discs (a) the effect of selected irrigant treatments on the mean percentages of dead (red), live (green) and unknown (blue) biovolumes, of the biofilms in their respective treatment groups and (b) the effect of irrigant treatment on the mean percentage of substratum coverage of the biofilms. **10**

coverage on discs (Figs 5 and 6), where the substratum coverage was more than that of control after irrigant treatment. Chlorhexidine (1.46 ± 0.42), trypsin (3.56 ± 1.18) and proteinase K (4.2 ± 1.01) with ultrasonics were significantly effective ($P < 0.05$) in reducing the substratum coverage as compared to saline with ultrasonics ($12\% \pm 4.9$) (Fig. 5b). The biofilm disrupting efficacy of the irrigants using tooth model biofilms is more apparent than the disc model in the representative images of the treated root canals (Fig. 6).

Discussion

The success of root canal treatment depends largely on chemomechanical procedures facilitating debride-

ment of the root canal system (Byström & Sundqvist 1983, Sjögren *et al.* 1997, Siqueira *et al.* 2002). The major cause of refractory endodontic infections is the persistence of bacteria in the root canal space (Moorer & Wesselink 1982). The biofilm communities of refractory endodontic infections are surviving under nutritionally stressful environmental conditions that possibly render them resistant to the chemomechanical disinfecting procedures (Siqueira 2002). A nutrient-stressed *in vitro* biofilm model was successfully established. As fermentation of glucose results in the production of acid, a low pH of broth supernatant was seen after an initial 7 days of regular medium replenishment. Leaving the biofilms for 7 days in the same medium led to the complete consumption of glucose from broth supernatant; thus, leaving the biofilm

Table 3 Anaerobic quantitative viable counts of all six sites taken from five biofilms each, in the nine experimental groups after their respective irrigant treatment. (1) upper half of root canal, (2) lower half of root canal, (3) medial portion of middle 1/3 of the artificially created lateral canal close to the main root canal, (4) distal portion of middle 1/3 of the artificially created lateral canal close to the outer surface of the tooth, (5) medial portion of apical 1/3 of the artificially created lateral canal close to the main root canal and (6) distal portion of apical 1/3 of the artificially created lateral canal close to the outer surface of tooth. The values are given as the mean number of bacteria (\pm standard error) as \log_{10} (CFU per sample)

Treatment Groups (Anaerobic)	1 Mean \pm SE	2 Mean \pm SE	3 Mean \pm SE	4 Mean \pm SE	5 Mean \pm SE	6 Mean \pm SE
Control	6.18 (0.08) **** *****	6.00 (0.03) **** *****	5.23 (0.31) ****	5.39 (0.05) ****	5.21 (0.44) *****	5.40 (0.31)
Saline + conventional	5.25 (0.81)	5.47 (0.12)	5.14 (0.22)	5.03 (0.27)	5.11a (0.14)	5.16 (0.25)
Saline + ultrasonics	4.63 (0.35)	5.39 (0.38)	5.03 (1.07)	4.93 (0.80)	4.15 (0.07)	4.86 (0.17)
Chlorhexidine + conventional	4.47a (0.66)	2.74 (0.34) * ****	3.25 (0.84) ****	3.86a (0.71) ****	2.55 (2.47) * ****	3.09 (2.99) * ****
Chlorhexidine + ultrasonics	3.25 (0.44) * **** *****	2.67 (2.08) * **** *****	1.94 (1.64) * **** *****	2.12 (0.60) * **** *****	1.88 (1.25) * **** *****	1.18 (1.54) * **** *****
Trypsin + conventional	5.87 (0.68) ***	5.15 (0.16) ***	5.16a (0.28) ***	4.82 (0.69) ***	4.62a (0.11) *** ****	4.89 (0.71) ***
Trypsin + ultrasonics	4.95 (0.21) ***	4.95 (0.68) ***	4.12 (0.71) *** ****	3.73 (0.52) *** **** *****	3.86 (0.37) *** **** *****	4.36 (0.42) *** **** *****
Proteinase K + conventional	5.43 (0.24) ***	5.11 (0.34) ***	4.55 (0.79) ***	4.83 (0.24) ***	4.44 (0.19) ***	3.97 (1.46) ***
Proteinase K + ultrasonics	5.30 (0.40) *** *****	5.24 (0.53) ***	4.69 (0.95) ***	4.6 (2.09) ***	4.56 (0.56) ***	4.48 (1.19) ***
NaOCl + conventional	0.30 (0.45) **	ND **	ND **	ND **	0.39 (0.76) **	0.51 (1.34) **
NaOCl + ultrasonics	0.45 (0.99) **	0.50 (1.27) **	0.26 (0.34) **	0.37 (0.68) **	0.32 (0.51) **	ND **

aUltrasonic irrigation better than conventional syringe irrigation using same irrigant ($P < 0.05$).

*Values significantly ($P < 0.05$) less than all other groups, except NaOCl.

**Values significantly less than all other groups ($P < 0.001$).

***No significant difference in the values between the groups ($P > 0.05$).

****Values significantly ($P < 0.05$) less than saline + conventional at the same sampling site.

*****Values significantly ($P < 0.05$) less than saline + ultrasonics at the same sampling site.

ND (not detected) = 0.00.

bacteria starved of the depleted fermentable carbohydrates. After elimination of glucose from the broth, to obtain energy, the biofilm bacteria started degrading

the proteins as was seen by SDS-PAGE results. The degradation of proteins results in the release of ammonia, which led to the rise in the pH of the broth

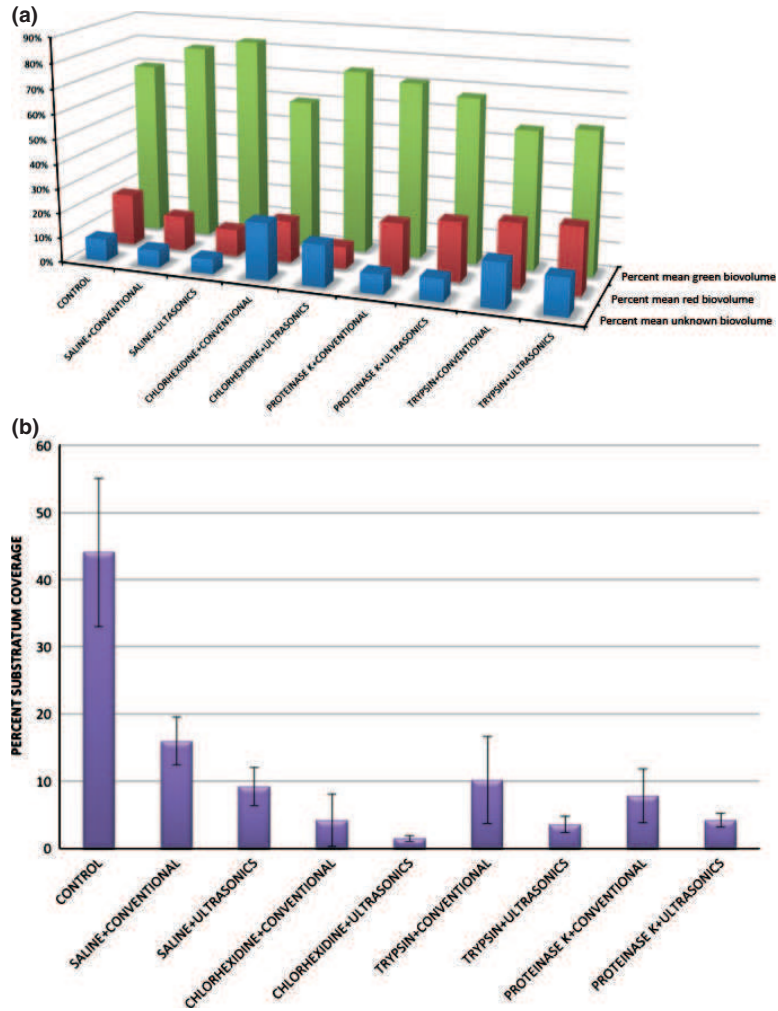


Figure 4 The effect of irrigant treatment on biofilms grown on teeth model (a) the effect of selected irrigant treatments on the mean percentages of dead (red), live (green) and unknown (blue) biovolumes, of the biofilms in their respective treatment groups and (b) the effect of irrigant treatment on the mean percentage of substratum coverage of the biofilms.

supernatant during the starvation period. The viable counts of the biofilm also decreased during the period of nutritional stress.

To test the biofilm killing capacity of selected chemical solutions and enzymic preparations, a study was carried out using the stressed *in vitro* biofilm model grown on hydroxyapatite discs. The results of the quantitative viable counts clearly showed that NaOCl was the most effective chemical in killing biofilm bacteria. This finding is consistent with previous studies where NaOCl was proven to be the most efficient irrigant (Byström & Sundqvist 1983, Siqueira *et al.* 1997). Trypsin and proteinase K were associated with greater bacterial killing than chlorhexidine and the

untreated control when the biofilm was cultured aerobically on FAA plates after treatment; however, when cultured aerobically on MacConkey agar and anaerobically on FAA, there was no significant difference between the killing efficacy of chlorhexidine, trypsin, proteinase K and untreated controls. It is probable that trypsin and proteinase K had an antibacterial effect on the aerobically growing organisms of the multispecies model biofilm (*i.e.* *E. faecalis*, *S. mitis*, *S. epidermidis* or *A. radidentis*). It is less likely that *E. faecalis* would have been affected by these enzymes, because MacConkey agar culturing did not yield any significant viability difference between the four irrigants. Therefore, these enzymes were more effective

in killing *S. mitis*, *S. epidermidis* and *A. radicans* whereas *E. faecalis* and *P. acnes* were more resistant.

Interestingly, in the hydroxyapatite disc model, chlorhexidine appeared to be inefficient in eradicating

the biofilm. This is in contrast to the existing literature (Evanov et al. 2004, Sena et al. 2006, Shen et al. 2009). This may be due to the resistance of the biofilm model developed during the nutritional stress phase.

The CLSM results of bacterial killing efficacy on disc model biofilm revealed that both trypsin and proteinase K had an effect, whereas there was no difference in chlorhexidine from the control group. The CLSM results seemed to be inconsistent with the case of anaerobic and MacConkey quantitative viable counts, where there was no significant difference between chlorhexidine, trypsin, proteinase K and the untreated control group. Previous studies using live–dead stain have revealed that staining with SYTO 9 and propidium iodide (PI) does not always produce distinct ‘live’ and ‘dead’ populations, whereas an intermediate cellular state is also observed (Christiansen et al. 2003, Hoefel et al. 2003, Berney et al. 2007). This is referred to in the manufacturer’s manual as ‘unknown’ (<http://probes.invitrogen.com/media/pis/>)

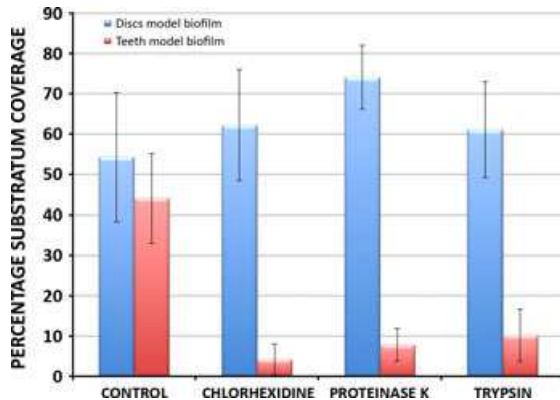


Figure 5 Comparison of the biofilm disrupting efficacy between disc model biofilm and tooth model biofilm using chlorhexidine, trypsin and proteinase K with conventional syringe irrigation.

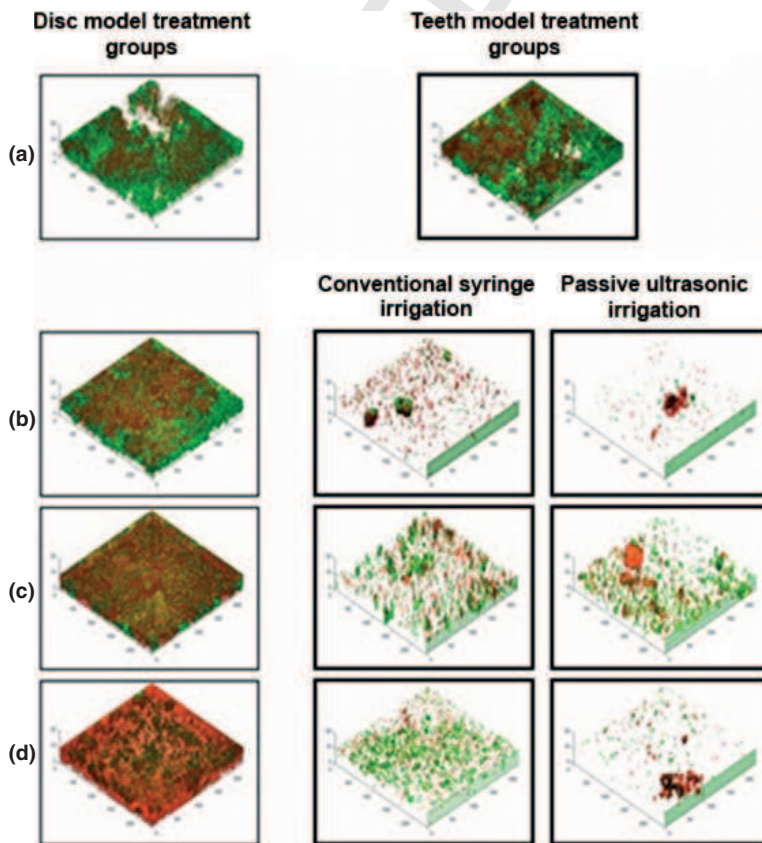


Figure 6 3-D view of the disc and teeth model biofilms. (a) control; (b) chlorhexidine; (c) proteinase K; and (d) trypsin.

mp34856.pdf) in which the partially damaged bacterial membranes can allow the penetration of red fluorescing PI dye even when the cells are viable. It is possible that these cells are capable of revival. The discrepancy between the cultural and CLSM results can be attributed to these intermediate cellular state bacteria. These were detected dead by CLSM results, but survived once plated and grown onto the rich FAA and MacConkey medium under appropriate growth conditions.

The bleaching effect of 1% NaOCl was confirmed on the SYTO 9 and PI dyes of live/dead stain using a planktonic *E. faecalis* suspension and *E. faecalis* single-species biofilm. Based on these findings, it was not possible to analyse these biofilms using CLSM imaging. Sodium thiosulphate has been used to neutralize NaOCl when assessing its effect on biofilms using **6** confocal microscopy (Bryce *et al.* 2009); however, further studies are needed to evaluate the effect of such compounds on bacterial biofilms.

Based on the findings of this initial study, a closed apex tooth model was used to determine the effects of these irrigants using conventional syringe irrigation and passive ultrasonic irrigation in the root canal space in the hope of making the findings more relevant. Every effort was made to make the tooth model as realistic as possible, although the flow of the irrigant within the root canal space remains different from that of a real root canal due to the inevitably imperfect seal of the two reapproximated root halves.

Bhuva *et al.* (2010), using a similar experimental model with a single-species biofilm, showed that sterile saline used as a control group was less effective as compared to NaOCl. The quantitative viable count results of the present study using a tooth model showed that NaOCl had the best biofilm killing efficacy with no significant difference when used with either ultrasonic irrigation or conventional syringe irrigation. Chlorhexidine with either conventional syringe or ultrasonic irrigation had significantly higher killing than saline. Moreover, chlorhexidine when used with ultrasonics achieved a biofilm killing significantly higher than those of both trypsin and proteinase K. The killing potential of proteinase K with or without ultrasonics and trypsin with conventional syringe irrigation was no different than those of saline. However, the results of both quantitative viable counts and CLSM showed that trypsin when used with ultrasonic activation had better killing as compared to saline with conventional or ultrasonic irrigation.

The CLSM results suggest that in the tooth model biofilm, even inactive saline was significantly effective in disrupting the biofilm and reducing the substratum coverage as compared to the untreated control group. This could be attributed to the mechanical flushing out of the biofilm from the root canal. However, the biofilm disruption of chlorhexidine, trypsin and proteinase K used with ultrasonics was significantly more than that of saline with ultrasonics.

Confocal laser scanning microscopy results revealed that chlorhexidine with ultrasonics proved to be the most effective approach for disrupting the biofilm. However, in contrast to their quantitative viable count results, the biofilm killing efficacy of this group was found to be lower than even untreated control, proteinase K and trypsin with conventional or ultrasonic irrigation. Another observation in this group was the high percentage of the unknown biovolume that was not differentiated by the *bioImage_L* software as either red or green during analysis. This suggests the possibility that the portion of this unknown biovolume may be the dead population left unrecognized during analyses resulting in inconsistent results of the quantitative viable counts and the CLSM.

In this study was tested the efficacy of two enzymes (proteinase K and trypsin) in disrupting and killing biofilm bacteria. Recent studies have shown that both these proteases were efficient in removing the biofilm of *S. aureus* 383 (Chaignon *et al.* 2007). Moscoso *et al.* (2006), using $100 \mu\text{g mL}^{-1}$ of both these **7** enzymes for 1 h, successfully demonstrated their inhibitory effects on biofilm development of *Streptococcus* **8** *toniae* (Moscoso *et al.* 2006). Patterson *et al.* (2006) used $100 \mu\text{g mL}^{-1}$ of proteinase K to determine the effect on biofilm viability. Moreover, it was also reported that a concentration as low as $32 \mu\text{g mL}^{-1}$ and $6.4 \mu\text{g mL}^{-1}$ of proteinase K and trypsin, respectively, effectively disrupted the mature biofilm of three different strains of *Gardnerella vaginalis* (Patterson *et al.* 2007). The enzymes have different substrate specificities: proteinase K endolytically cleaves the peptide bonds of aliphatic, aromatic or hydrophobic amino acids, whereas trypsin is specific for the peptide bonds of lysine and arginine (Chaignon *et al.* 2007). In the present study, trypsin as well as proteinase K with ultrasonic activation was efficient in disrupting the biofilm although less than chlorhexidine. This may be attributed to the combined mechanical effect of ultrasonics complemented with the hydrolytic activity of these proteases by acting on the protein fraction of the biofilm. Although there is little evi-

dence on the exact antibacterial effects of these enzymes, in this study, trypsin especially when used with ultrasonics caused bacterial killing when compared to saline. It is possible that the protease acts by degrading the protein components of the bacterial cell walls and membranes, causing cellular damage and ultimately cell lysis and death. Furthermore, the proteolytic enzymes might disrupt the extracellular matrix, which is also made of proteins secreted by the bacteria, thus reducing the cohesion of the biofilm. Recent studies suggest that cell surface proteins might act as co-aggregation factors during the biofilm formation as these surface structures were found to be protease sensitive (Furukawa *et al.* 2011). Nonetheless, the killing efficacy of the two proteases was lower than that of NaOCl.

For all irrigants except NaOCl, the results of quantitative viable counts as well as CLSM analysis showed that passive ultrasonic was more effective than conventional syringe irrigation. Previous studies suggested that the heat generation by ultrasonics (Zeltner *et al.* 2009) may also have an enhancing effect on the antibacterial properties of chlorhexidine (Evanov *et al.* 2004) and NaOCl (Cunningham & Balekjian 1980, Abou-Rass & Oglesby 1981, Cunningham *et al.* 1982). The heat generated by ultrasonics may also have enhanced the antibacterial properties of trypsin.

Greater biofilm disruption by flushing out and shear stress is achieved in the tooth model as compared to the disc model biofilm because the mechanical effects of irrigation protocol are better simulated in a confined root canal space. Moreover, this tooth model offers the potential advantage to allow the investigation of the efficacy of different endodontic instrumentation techniques.

Conclusion

This study suggests that trypsin used as an irrigant with ultrasonic activation has biofilm killing and disrupting potential. Even though the antibacterial properties were not as good as NaOCl, it certainly has a biofilm disrupting ability. Further studies are necessary to assess its compatibility with other irrigants and to determine its potential in a disinfection regimen, where enzymes can help disrupt the biofilm and render single bacterial cells more susceptible to killing by other stronger antimicrobials. Investigations are also required to determine its impact and toxicity on dentine and periapical tissues.

Acknowledgments

We acknowledge support from the Higher Education Commission of Pakistan; from the Dental Institute, King's College London. We thank Professor Gunnar Dahlén (Department of Oral Microbiology, Faculty of Odontology, University of Göteborg, Sweden) for providing the *E. faecalis* strain and Dr. Luis Chevaz de Paz (Department of Oral Biology, Malmö University, Sweden) for providing *BioImage_L* software.

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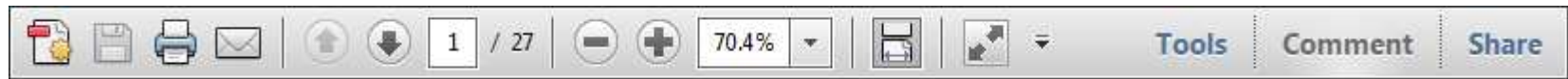
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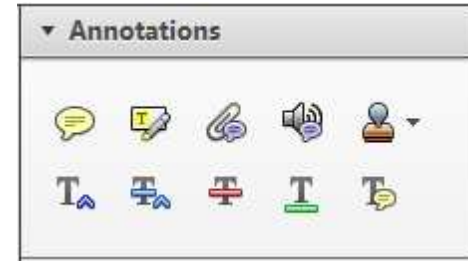
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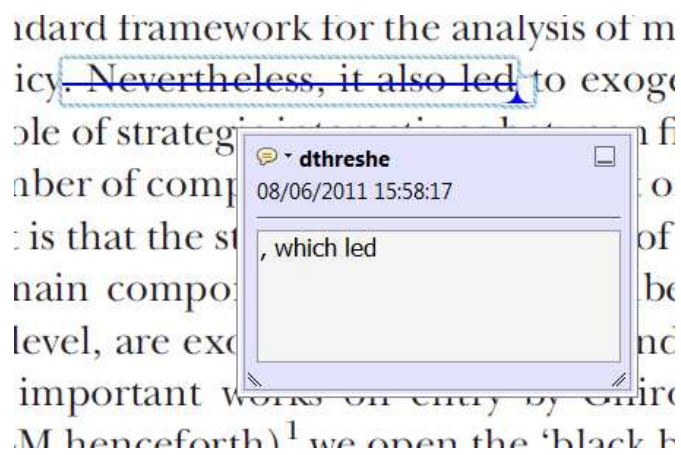
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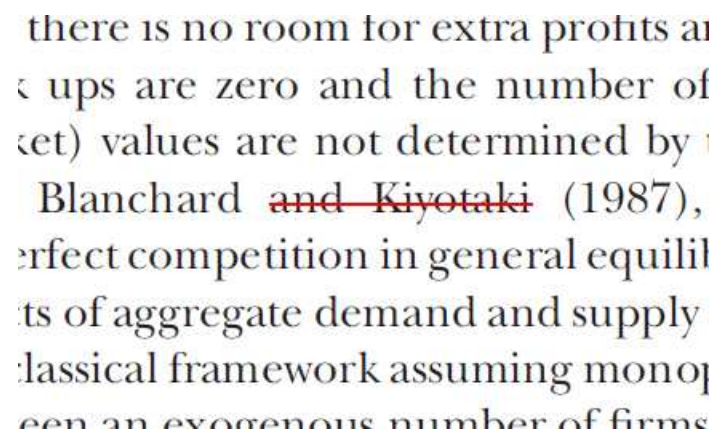
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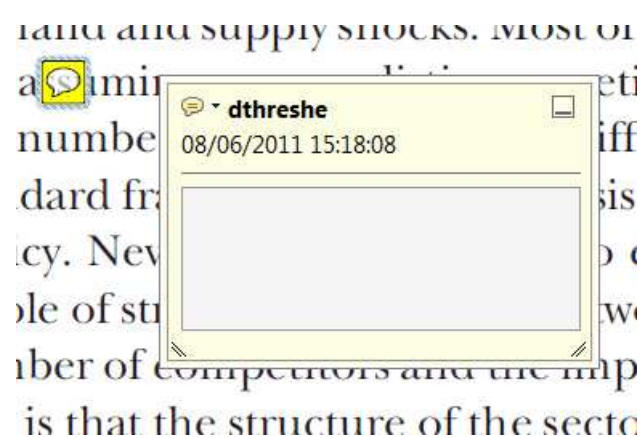
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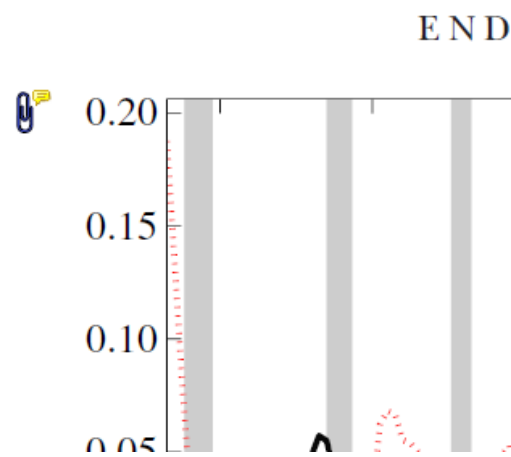
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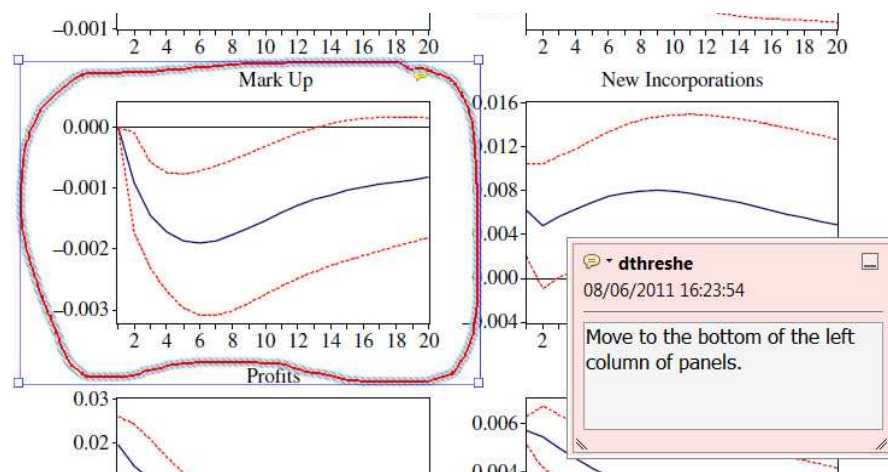


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