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An *in vitro* and *ex vivo* study on two antibiotic-based endodontic irrigants: a challenge to sodium hypochlorite

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SUMMARY

Amongst the bacterial species which most often cause endodontic failures, *Enterococcus faecalis* is the most important. This study compared the effectiveness of sodium hypochlorite and two new generation antibiotic-based endodontic irrigants, Tetraclean and MTAD. By means of an *in vitro* agar dilution assay, we show that both Tetraclean and MTAD are 100% effective against 54 clinical isolates at dilutions up to 1:256 and 1:1048, respectively, whereas sodium hypochlorite completely loses its effectiveness when diluted more than 32 times. The bactericidal effect of both Tetraclean and MTAD can be ascribed not just to their antibiotic component per se, but also to a synergistic effect among the several ingredients included in the formulations. Moreover, by an *ex vivo* model of teeth extracted and experimentally infected with *E. faecalis* ATCC 29212, we show that both the antibiotic-based endodontic irrigants are effective in eliminating bacterial cells in 93 to 100% of the test samples. The results of these pre-clinical studies strongly support a wider use of this new group of endodontic irrigants in daily clinical practice.

KEY WORDS: Tetraclean, MTAD, *Enterococcus faecalis*, Sodium hypochlorite, Endodontic irrigants

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INTRODUCTION

Notwithstanding the advances in the treatment of endodontic diseases, the occurrence of failures remains a major problem in clinical practice. The need for retreatment may be due either to reinfection by oral bacteria or, more often, to the persistence and regrowth of microrganisms not eliminated during the previous treatment. The anatomical complexity of the root canal system lends itself to shelter bacteria. *Streptococci* and some Gram-positive rods, such as *Actinomyces* and *Lactobacillus* spp., appear to invade the dentinal tubules better than several Gram-negative species (Ørstavik and Haapasalo, 1990; Love, 2001; Peters *et al.*, 2000; Matsuo *et al.*, 2003). Moreover, Gram-positive facultatively anaerobic bacteria, which best tolerate harsh ecological conditions, can survive more easily. It has also been shown that their relative proportion is increased after instrumentation and disinfection procedures, when the overall number of bacteria is strongly reduced (Peciuliene *et al.*, 2001; Chavez De Paz *et al.*, 2003). It is well documented that *Enterococcus faecalis* is the dominant microorganism in persistent apical periodontitis (Peciuliene *et al.*, 2001; Molander *et al.*, 1998; Peciuliene *et al.*, 2000; Hancock *et al.*, 2001).

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Recently, the new diagnostic approaches of molecular biology confirmed that *E. faecalis* is one of the species most commonly detected in cases of primary endodontic infections (Roças *et al.,* 2004a; Roças *et al.,* 2004b). Furthermore *E. faecalis* accounts for up to 77% of therapeutic failures (Siqueira and Roças, 2004; Siqueira and Roças, 2005).

The current methods of root canal cleaning and shaping produce a smear layer (McComb and Smith, 1975; Moodnick *et al.*, 1976; Mader *et al.*, 1984; Cengiz *et al.*, 1990) containing inorganic and organic substances, microrganisms and necrotic material (Pashley, 1992). The use of endodontic irrigants is meant to properly clean the root canal system (Jeansonne and White, 1994; Kuruvilla and Kamath, 1998), possibly through removal of the smear layer itself (Williams *et al.*, 1995) and, more important, to grant microbial clearance. One of the most used, yet widely debated, endodontic irrigants is sodium hypochlorite (Siqueira *et al.*, 1997). In spite of its advantages (broad antimicrobial spectrum, strong and fast oxidizing ability, easy to use, cheap, etc.), it suffers from several drawbacks like unpleasant odour and taste, and, most of all, aggressiveness versus host soft tissues (Ehrich *et al.*, 1993; Türkün *et al.*, 1998; Hülsmann and Hahn, 2000). Shih *et al.* (1970) demonstrated that *E. faecalis* and *Staphylococcus aureus* strains were easily killed when *in vitro* directly treated with sodium hypochlorite at a dilution as low as 1:1000 while, in a model of infected teeth, even full strength sodium hypochlorite failed to eradicate the bacteria.

Accordingly, we added insights on the poor effectiveness of sodium hypochlorite in an *ex vivo* model of extracted and experimentally infected teeth. We showed that besides a large reduction in bacterial load, occurring immediately after irrigation with sodium hypochlorite (5.25%), a dramatic rebound was detected after a few days (Rimoldi *et al.*, 2006; Neglia *et al.*, 2008). Both experimental and clinical data support the conclusion that the poor effectiveness of sodium hypochlorite may be ascribed, first, to its inactivation by dentin, collagen and other organic compounds present in the necrotic root canal, and, second; its poor capacity of penetrating dentinal canaliculi cause of its high surface tension (Giardino *et al.*, 2006). To overcome these clinical problems, the use of antibiotic-based irrigants is proposed because of their higher penetrating power, coupled with a substantivity or residual effect, namely the capability to be absorbed by the dental tissues and to be released gradually and for prolonged periods of time. On this basis, a few years ago, MTAD, an antibiotic-based irrigant, was described and later introduced on the market (Torabinejad *et al.*, 2003). This irrigant is an aqueous solution made up of doxycycline, a surface-active agent and citric acid. More recently, our group reported another antibiotic-based endodontic irrigant, Tetraclean, whose effectiveness has been recently described (Neglia *et al.*, 2008; Giardino *et al.*, 2006).

In the present study, the *in vitro* effectiveness of sodium hypochlorite, Tetraclean and MTAD was investigated on 54 *E. faecalis* clinical isolates by means of an agar dilution assay. In addition, using the recently described (Neglia *et al.*, 2008) *ex vivo* model of decoronated and experimentally infected human teeth, we compared the irrigation efficacy of both Tetraclean and MTAD. The overall results show that the new generation antibiotic-based endodontic irrigants are very promising for the achievement of a more predictable and complete elimination of endodontic infection.

MATERIALS AND METHODS

A group of 54 clinical isolates of *E. faecalis* were kindly provided by the Microbiology and Virology Laboratories of the Policlinico Hospital in Modena and employed in this study. Moreover, according to the specific protocols employed in this study, several ATCC strains were used as controls: *E. faecalis* 29212, *S. aureus* 25923, *E. coli* 25922, *E. hirae* 10541, *S. aureus* 6538P, *P. aeruginosa* 15442, *E. coli* 10536 and *E. coli* 13762. Sodium hypochlorite was purchased from Incofar (Modena, Italy).

Tetraclean, recently described (Neglia *et al.*, 2008; Giardino *et al.*, 2006), was kindly provided by Dr. Giardino and BioPure™ MTAD, simply referred to as MTAD, was purchased from Dentsply Tulsa Dental (OK, USA); both irrigants were reconstituted with their respective diluents. All the irrigants were employed either undiluted or serially two-fold diluted in Mueller-Hinton Agar medium

(MHA - Oxoid, Hampshire, U.K.). Prior to use, all the irrigants were sterilized by filtration with 0.45 µm filters (Schleicher & Schuell, Germany). Doxycycline Hyclate was provided by Sigma and Component C by Dr. Giardino. Tryptone Soya Broth (TSB - Oxoid, Hampshire, U.K.) was used for bacterial cultures from frozen stocks. Brain Heart Infusion (BHI; Difco, Detroit, MI) was employed for the infection, irrigation and further incubation of the teeth in the *ex vivo* studies. Bile Aesculin Agar (BAA - Oxoid, Hampshire, U.K.) was used to evaluate the bacterial growth from tooth samples at time points after irrigation. For the *ex vivo* study, 38 freshly extracted monoradicular human healthy teeth were used. These teeth, which had been extracted for malposition or orthodontic reasons at the School of Dentistry of Modena, were selected on the basis of their relative dimensions and morphological similarities. Patients volunteered to donate their extracted teeth for research purposes.

Prior to each experiment, the microrganisms (stored at -20°C in 1 ml aliquots of TSB and 5% glycerol solution - BDH - v/v) were thawed, suspended in 9 ml of TSB and incubated overnight at 37°C. The broth cultures were then plated on selective media and single colonies were harversted after 24 hours, suspended in BHI and allowed to grow overnight at 37°C. By spectrophotometric reading at λ=625 nm, bacterial concentration was estimated and then adjusted at the desired CFU/ml.

In vitro studies

The dilution agar test was employed to assess the enterococcal susceptibility to the irrigants, according to the NCCLS M7-A4 (1997). This test evaluates the minimal inhibiting concentration (MIC). Briefly, Petri dishes were prepared with MHA and serial two-fold dilutions of every test compound. In detail: 5.25% sodium hypochlorite was diluted up to 1:512; Tetraclean and MTAD were serially diluted up to 1:65536. Doxycycline was tested at concentrations ranging from 256 µg/ml (corresponding to its concentration in Tetraclean 1:40 and in MTAD 1:120 dilutions) to 0.5 µg/ml (corresponding to its concentration in Tetraclean 1:20000 and in MTAD 1:60000); the Component C was tested at concentrations ranging from 2 mg/ml (corresponding to undiluted Tetraclean) to 0.5 µg/ml (i.e. the concentration in Tetraclean diluted 4096 times). In each assay, 3 MHA plates with no irrigants were used for assessment of positive controls. The bacterial suspension was adjusted to 10^7 CFU/ml in saline and inocula of 10^4 CFU/spot were applied onto the agar surface using a Steers replicator. The plates were aerobically incubated at 35°C and read after 20-22 hours of incubation; the lowest compound concentration completely inhibiting the visible growth of the tested microorganisms was determined as MIC. The test was performed, in triplicate, in three different experiments. The adequate control strains were included in every MIC determination test.

Ex vivo studies

After teeth extraction, debris, calculus and soft tissue remnants on the root surfaces were removed using a Gracey curette and all the teeth were placed in saline solution. The crowns were flattened using diamond steel discs (Brasseler USA, Savannah, GA) and a final length of 15 mm was achieved for each tooth. Root canal instrumentation was performed using Niti K3 endodontic instruments (SybronEndo Orange, CA, USA) up to the end of the root canal with a 30/.06 file. The root canals were irrigated with 1 ml of 5.25% sodium hypochlorite, between each file change. The samples were finally dried with sterile paper points, immersed in 1 ml BHI and autoclaved at 121°C for 30 minutes.

After this step, they were handled in asepsis under laminar flow. Prior to use, all the teeth were incubated at 37°C for 24 h to exclude viable residual bacteria and, in particular, 3 of them were maintained for all the duration of the experiments as long term negative controls. The remaining 35 teeth were infected by a 24 hours immersion in BHI containing *E. faecalis* ($5x10^5$ CFU/ml; 2 ml/tooth), according to a previously described protocol (Neglia *et al.*, 2008; Siqueira *et al.*, 1997; Onçag *et al.*, 2003).

The efficiency of dentinal tubules infection by this model had been previously established (Neglia *et al.*, 2008; Shabahang and Torabinejad, 2003; De Almeida Gomes *et al.*, 2006). The infected teeth were then randomly divided into 3 groups and treated with sterile BHI (5 teeth), Tetraclean (15 teeth) or MTAD (15 teeth). The protocol of treatment consisted in:

a) slow injection of either irrigant or BHI (4 ml)

into the root canals using a sterile endodontic syringe;

b) 5 minutes immersion of each tooth in 2 ml of fresh irrigant or BHI, pre-heated at 37°C in order to achieve the best efficiency (Rimoldi *et al.*, 2006; Neglia *et al.*, 2008);

c) incubation in fresh BHI (2 ml/tooth) at 37°C for 23 days.

After irrigation, at various time-point intervals of 24-48 hours, 300 µl were collected from each tube, appropriately diluted and plated in duplicate onto BAA (100 µl/plate) to determine the bacterial counts. Every 300 µl withdrawal was always immediately replaced with the same volume of fresh BHI. The plates were incubated for 24 hours at 37°C and then the CFUs were recorded. The CFU values were taken as a measure of the number of viable bacteria present in each tooth specimen (Neglia *et al.*, 2008). The efficacy (%) of each irrigant was calculated as follows:

% Efficacy = [100 - (number of culture positive teeth / total number of irrigated teeth)].

Statistical analysis was carried out by chi-squared test in order to assess the differences in the bactericidal activity between the whole irrigants and each single component. The chi-squared test was also employed to evaluate the significance of the results obtained by the *ex vivo* model of extracted, infected and irrigated human teeth. The level of significance was set at 0.05 for all the analyses.

RESULTS

An *in vitro* agar dilution assay was performed to test the susceptibility of 54 clinical isolates of *E. faecalis* to sodium hypochlorite, Tetraclean and MTAD. The results, depicted in Figure 1, show that 100% of the strains were inhibited by sodium hypochlorite up to 1:16 dilution, while a complete loss in effectiveness was observed at a 64 times dilution. By contrast, 100% of the clinical strains were still inhibited by Tetraclean diluted 1:256, whereas 4 clinical isolates (7.4%) were still sensitive to the 1:65536 dilution. Figure 1 also shows the results for MTAD: 100% of the clinical strains were still inhibited by MTAD diluted 1:1024, whereas 11.1% (6 clinical isolates) were still sensitive at the 1:65536 dilution.

In order to investigate the role of doxycycline with respect to each whole irrigant, we assessed the antibiotic MIC for the 54 strains. In parallel, control strains were also tested. The results, reported in table 1, show that the MIC for all the strains was 64 µg/ml; 22% of the isolates were doxycycline resistant, since they were able to grow at 16 µg/ml. Moreover, 15% of the strains were sensitive, showing a MIC ≤1 µg/ml. The control strains displayed the expected MIC (data not shown). Figure 2 compares the results obtained with each whole irrigant and doxycycline alone, the latter being assessed at the concentrations corresponding to the doxycycline content included in the irrigants' dilutions. As emphasized by the framed

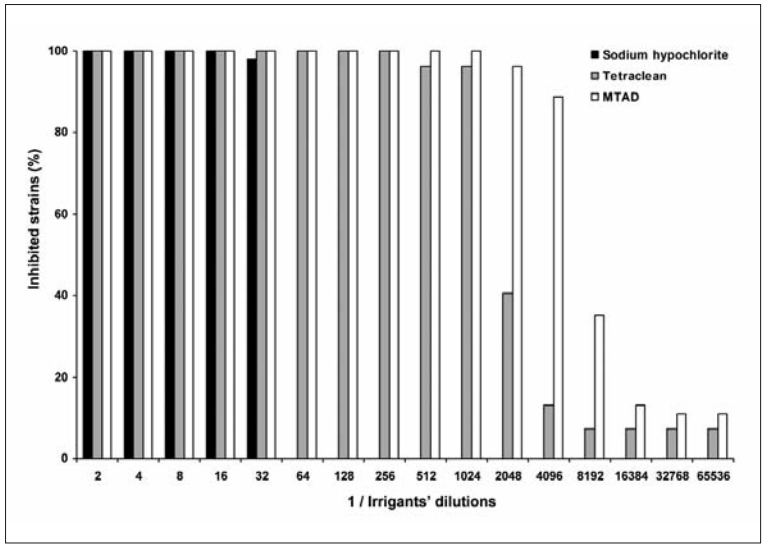

FIGURE 1 - *In vitro dose-dependent bactericidal activity of sodium hypochlorite, Tetraclean and MTAD. Group of 54 E. faecalis clinical isolates were assessed for susceptibility to each of the irrigants, by means of the agar dilution assay according to the NCCLS M7-A4 procedure (31). Briefly, inocula of a bacterial suspension (10^4 CFU/spot) were applied, by a Steer replicator, onto the surface of agar plates containing MHA plus serial dilutions of each irrigant. After a 24 h incubation at 37°C, the growth was recorded and the results expressed as percentage of inhibited strains were determined for each irrigant.*

TABLE 1 - *Doxycycline MIC of 54 clinical isolates of E. faecalis as assessed by dilution agar test.*

Doxycycline concentration (μg/ml)	*Inhibited strains (%)*
256-64	100
32.0	96
19.5	83
16.0	78
14.7	74
9.8	50
8.0	31
7.3	30
4.9	20
4.0	15
2.0	15
1.0	15
0.5	11

Doxycycline MIC: ≥16 μg/ml resistant; ≤1 μg/ml susceptible

areas, the differences in efficacy occurred within Tetraclean dilutions of 1:512 and 1:1024 and at MTAD dilutions ranging between 1:2048 and 1:4096. At such dilutions, the efficacy of each irrigant was significantly ($p<0.05$) higher than that of the antibiotic tested alone.

Since the Tetraclean formulation has been recently improved by addition of a further chemical (the Component C), its efficacy was assessed too. The results, summarized in Table 2, showed that Component C was fully effective in preventing bacterial growth up to 125 μg/ml, whereas it became totally ineffective at 2 μg/ml (corresponding to Tetraclean diluted 1:16 and 1:1024, respectively).

In order to further compare the efficacy of Tetraclean and MTAD, we used the previously described *ex vivo* model of experimentally infected and irrigated human teeth (Neglia *et al.*, 2008). As shown in Figure 3, an immediate and drastic drop to undetectable values of CFUs (<10 CFU/ml) was evident in the 15 teeth irrigated with Tetraclean as soon as 24 hours post irrigation. In some cases, however, a transient and low level positivization was observed. At later time points,

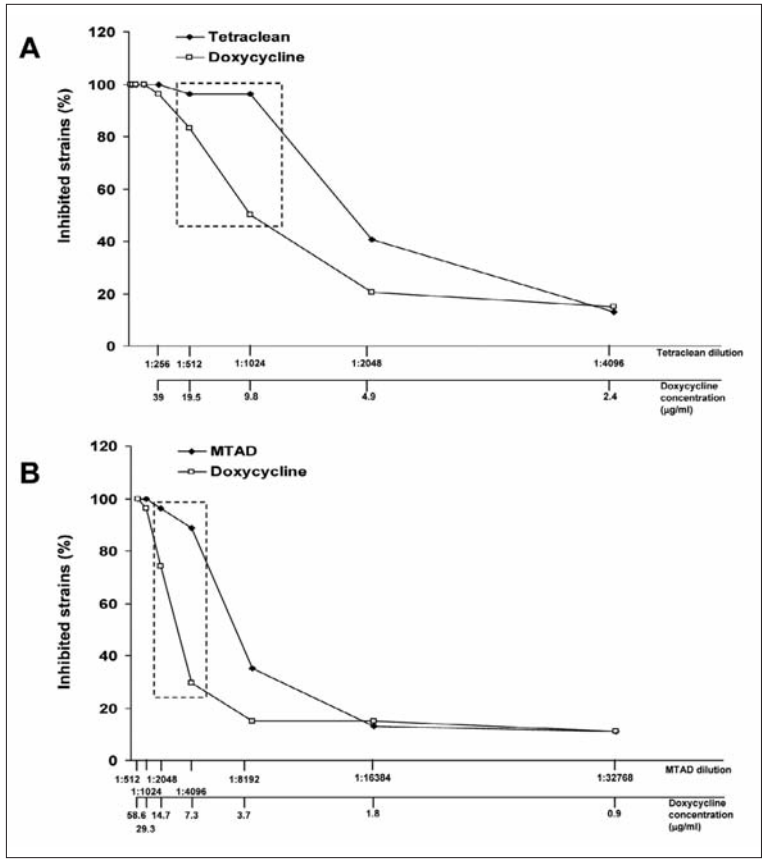

FIGURE 2 - *Direct comparison between the effectiveness of doxycycline alone and Tetraclean (Panel A) or MTAD (Panel B). The 54 E. faecalis clinical isolates were assessed for susceptibility to each of the irrigants or to doxycycline alone, by means of the agar dilution assay according to the NCCLS M7-A4 procedure (31). Briefly, inocula of a bacterial suspension (10^4 CFU/spot) were applied, by a Steer replicator, onto the surface of agar plates containing MHA plus serial dilutions of each irrigant or doxycycline. After a 24 h incubation at 37°C, the growth was recorded and the results were expressed as percentage of inhibited strains. For each chart, two X-axes are depicted: the top one reports the irrigants' dilutions; the bottom one reports the doxycycline concentrations. Each irrigant dilution contains the same doxycycline concentration indicated in the corresponding bottom X-axis.*

TABLE 2 - *Component C MIC of 54 clinical isolates of E. faecalis as assessed by dilution agar test.*

Component C concentration (μg/ml)	*Inhibited strains (%)*	*Corresponding Tetraclean dilutions*
2000-125	100	1:16
62.5	98	1:32
31.3	87	1:64
15.7	85	1:128
7.8	80	1:256
3.9	41	1:512
2.0	0	1:1024
1.0	0	1:2048
0.5	0	1:4096

no bacterial growth was ever detected. In the group of teeth irrigated with MTAD (Figure 4), a different trend was observed: the bacterial counts dropped more gradually but steadily. At the end of the experiment (i.e. 528 hours post-irrigation), one single specimen kept on giving positive but small values of bacterial CFUs. In parallel control groups, irrigation with BHI produced the expected 5 logarithms reduction in CFU; by the following time point, the CFU rapidly rose and steadily levelled to approximately 10^9/ml throughout the duration of the experiment (data not shown).

The overall efficacy of the two irrigants, as measured 528 hours after irrigation, was therefore 100% for Tetraclean and 93.3% for MTAD (Table 3). This difference in efficacy was not statistically relevant, as assessed by the chi-squared test ($p>0.05$).

DISCUSSION

By *in vitro* and *ex vivo* studies here we show that, when compared to sodium hypochlorite, both Tetraclean and MTAD display a greater antimicrobial efficacy.

When assessing the effectiveness of any endodontic irrigant, *E. faecalis* is often chosen as a model since this species is the main responsible of endodontic failures (Molander *et al.*, 1998; Heath *et al.*, 1996). Notoriously, *E. faecalis* is more resist-

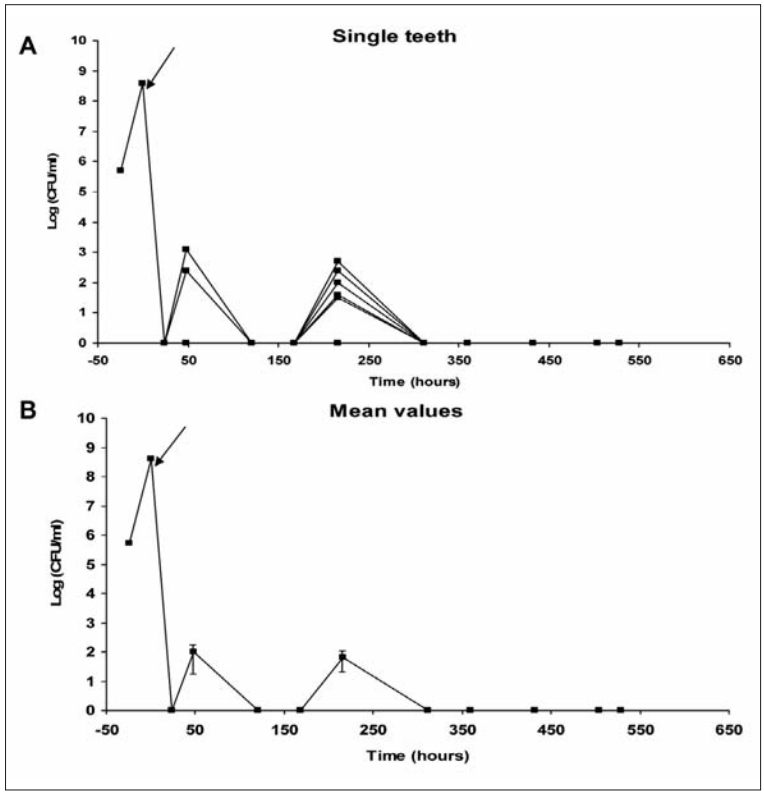

FIGURE 3 - *Effectiveness of Tetraclean irrigation in extracted and experimentally infected teeth. Human teeth, prepared as detailed in Materials and Methods, were infected by immersion in a suspension of E. faecalis ATCC 29212 (10^5 CFU/ml). After 24 h incubation, teeth were irrigated with Tetraclean and then incubated at 37°C in fresh BHI (2 ml/tooth). At the indicated times, aliquots from each sample were plated in BAA to determine the bacterial load; the results were expressed as log (CFU/ml). Panel A shows a kinetic of the results obtained in the single teeth. Panel B shows the mean values relative to all the 15 teeth. The bars depict the standard error of the mean. The arrows point the time of irrigation.*

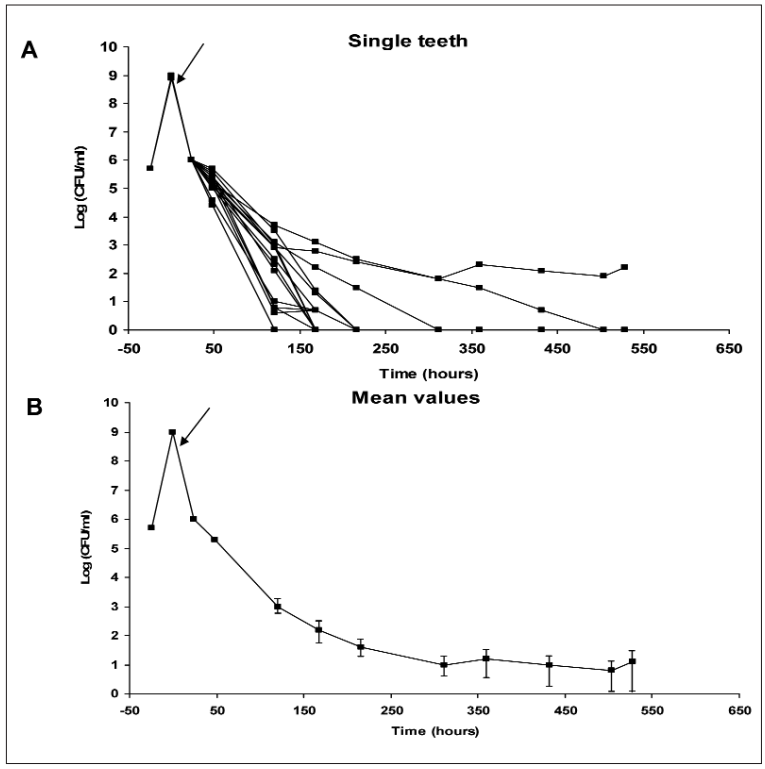

FIGURE 4 - *Effectiveness of MTAD irrigation in extracted and experimentally infected teeth. Human teeth, prepared as detailed in Materials and Methods, were infected by immersion in a suspension of E. faecalis ATCC 29212 (10^5 CFU/ml). After a 24 h incubation teeth were irrigated with MTAD and then incubated at 37°C in fresh BHI (2 ml/tooth). At the indicated times, aliquots from each sample were plated in BAA to determine the bacterial load; the results were expressed as log (CFU/ml). Panel A shows a kinetic of the results obtained in the single teeth. Panel B shows the mean values relative to all the 15 teeth. The bars depict the standard error of the mean. The arrows indicate the time of irrigation.*

TABLE 3 - *Tetraclean and MTAD efficacy as assessed by the ex vivo model of infected and irrigated teeth.*

Irrigant	*Culture positive teeth/Irrigated teeth (528 h post-irrigation)*	*Efficacy (%)*
Tetraclean	0/15	100
MTAD	1/15	93.3

ant to disinfecting agents than other endodontic microorganisms (Haapasalo and Orstavik, 1987; Gomes *et al.*, 1996). It has been shown to penetrate the dentine even in the presence of smear layer, it is ecologically tolerant and it can survive in the obstile environmental conditions created by the chemomechanical treatment (Figdor *et al.*, 2003). In a recent *in vitro* study employing *E. faecalis* ATCC 29212 (Neglia *et al.*, 2008), we showed that a contact time as short as 5 minutes with sodium hypochlorite was sufficient to kill all the bacteria. By means of the agar dilution test, we here demonstrate that sodium hypochlorite inhibits the growth of all the clinical strains tested and that its efficacy is retained up to 16-fold dilution. By contrast, both Tetraclean and MTAD are 100% effective in preventing bacterial growth even when 256- and 1024-fold diluted, respectively. Unlike sodium hypochlorite, Tetraclean and MTAD retain some activity even at the highest dilutions (approximately 10% of the strains were still susceptible). Furthermore, the observed differences of 2-3 fold dilutions in dose-response between Tetraclean and MTAD may be ascribed to their different content in doxycycline (3 times more in MTAD than in Tetraclean). With the aim to ascertain the specific role of doxycycline, a direct comparison between each of the whole irrigants and the antibiotic alone, taken at the corresponding concentrations, was performed. The results indicate that both irrigants are more effective than doxycycline alone. The statistically significant differences, observed within a precise range of doxycycline concentrations (20 µg/ml to 7 µg/ml), imply that the action of the two irrigants is not simply due to the presence of the antibiotic, but is also the result of a synergistic effect among the various components. It follows that as 16 µg/ml is the cut-off value for the resistance to doxycycline, the use of the irrigant will kill bac-

teria at antibiotic concentrations otherwise ineffective. Moreover, the fact that the component C exerts some antibacterial activity underlines its relevance in the overall efficacy of the Tetraclean new formulation.

In order to further investigate the efficacy of Tetraclean and MTAD, we employed an already described *ex vivo* model of extracted and experimentally infected human teeth in which the bacterial load was assessed at various times after irrigation (Neglia *et al.*, 2008; Shabahang and Torabinejad, 2003; De Almeida Gomes *et al.*, 2006). Both irrigants show an overall very high and similar efficacy, notwithstanding some differences in the kinetics of action. In particular soon after irrigation, MTAD shows a drop of about 3 logarithms in the number of the CFUs and, at later time-points, such number further drops gradually and steadily to undetectable levels for all but one tooth.

Interestingly, Tetraclean causes an immediate drop to undetectable CFUs soon after irrigations in all the samples that remain negative for all the time points tested but two, when few teeth cultures become positive. Notwithstanding such differences, the overall long-term efficacy of both irrigants is 93.3-100%.

This finding profoundly differs from what was previously observed when assessing sodium hypochlorite as irrigant in the same *ex vivo* model (Neglia *et al.*, 2008). In that study, the irrigation with sodium hypochlorite could abate microbial load to undetectable levels, but such effect was transient and, at the end of the experiment, not less than 70% of the treated samples showed bacterial regrowth. Those findings were ascribed to the complexity of tooth anatomy, together with the weak penetrating power of sodium hypochlorite (Giardino *et al.*, 2006). Vice versa, the present findings, describing the gradual and long-lasting effect of Tetraclean and MTAD, strengthen the usefulness of such antibiotic-based irrigants. In this respect, the following issues should also be taken into account. First, tetracyclines are effective against a wide range of microorganisms including most clinical isolates of *E. faecalis*, whose intrinsic resistance to various antibiotics has been reported (Portenier *et al.*, 2003). Second, citric acid is known to remove the smear layer that covers the root canal surface and/or plugs the entrance to the dentinal tubules (Haznedaroğlu and Ersev, 2001). Third, the surfactants included in Tetraclean and MTAD formulations may facilitate the penetration of the medicament (Giardino *et al.*, 2006).

This last feature makes it possible for doxycycline to reach those bacteria that survive within the dentinal tubules and represent an important reservoir of reinfection after therapy. A successful endodontic treatment is therefore dependent on the initial killing of all the bacteria, i.e. those present in the root canal as well as those already penetrated in depth. The achievement of microbicidal doses becomes critical in the endodontic environment, because in such harsh conditions bacteria may aggregate to form a biofilm or enter a stationary phase, thus acquiring a resistant phenotype (Love, 2001; Figdor *et al.*, 2003; Svensäter and Bergenholtz, 2004). Accordingly, in endodontic therapy the local use of antibiotics allows the use of the necessary very high concentrations. Moreover, tetracyclines may represent the optimal choice to grant long-lasting antimicrobial effects, since they readily attach to dentine and are gradually released, retaining their antibacterial activity (Stabholz *et al.*, 1993; Khademi *et al.*, 2006).

In the present study, we could observe transient and single episodes of positivization in some of the Tetraclean-irrigated teeth. In particular, the 2 teeth samples that show some bacterial growth at 48 hours post irrigation (teeth T4 and T5) are different from the 6 samples that show a later bacterial growth (T2, T8, T10, T11, T12 and T14 become positive at 216 hours). We may envisage that such phenomena are due to the few bacteria that had survived the initial irrigation, started to regrow and are, in turn, wiped out by the gradual release of the antibiotic.

In any case, irrespective of the reasons of such transient events, it is important to underline that, at later time points and at the end of the experiment, 100% of efficacy was recorded for Tetraclean.

Overall, by *in vitro* and *ex vivo* studies, we provide evidence of the high efficacy of two antibiotic-based endodontic irrigants with respect to sodium hypochlorite whose usefulness should be carefully reconsidered, in parallel with the introduction of new generation antibiotic-based endodontic irrigants in the daily clinical routine.

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