Comparative *in vitro* and *ex vivo* studies on the bactericidal activity of Tetraclean, a new generation endodontic irrigant, and sodium hypochlorite

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SUMMARY

The aim of this study was to compare the efficacy of a new generation endodontic irrigant, Tetraclean, to the widely used sodium hypochlorite. Tetraclean combines a powerful detergent effect with a strong antimicrobial efficacy, whereas sodium hypochlorite has several drawbacks and is sometimes ineffective in preventing microbial-mediated endodontic failure. The bactericidal activity of both irrigants against *Enterococcus faecalis*, the most commonly isolated species from root canals of teeth with post-treatment disease, was assessed i) *in vitro,* according to the European Standard lines for the evaluation of the bactericidal activity of chemical disinfectants, and ii) with an *ex vivo* model of extracted and decoronated human teeth, infected with *E. faecalis* and subsequently irrigated with either of the irrigants. Both irrigants display very similar bactericidal activity against *E. faecalis in vitro*. However, the *ex vivo* model shows that only in the teeth irrigated with Tetraclean did the bacterial burden gradually drop until no bacteria were detectable a few days post-irrigation. Vice versa, in the teeth irrigated with sodium hypochlorite, the drop in the bacterial burden was rapid but temporary and most of the teeth were colonized again by 48 hours post-irrigation.

KEY WORDS: Endodontic irrigant, *Enterococcus faecalis*, Root Canal System (RCS), Sodium hypochlorite, Teeth, Tetraclean

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INTRODUCTION

The main target of endodontic therapy is to remove bacteria from root canal and dentinal tubules. Mechanical debridement of infected root canals significantly reduces bacterial contamination (Siqueira *et al.*, 1999). Nevertheless, the instrumentation techniques currently employed leave many areas of the root canal system (RCS) unaffected. Therefore, the use of a root canal irrigant is normally required for disinfection of the RCS. Sodium hypochlorite is the most common irrigant employed in endodontics to date, notwithstanding its numerous drawbacks like unpleasant odour and taste and, even more relevant, the high toxicity displayed when it is extruded into the periapical tissues (Spangberg *et al.*, 1973). The use of sodium hypochlorite as an endodontic disinfectant also has several limitations: it does

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not remove the smear layer (McComb and Smith, 1975), nor does it provide a complete disinfection of the RCS (Shuping *et al.*, 2000). Many root canals, infected *in vitro* by *Enterococcus faecalis*, have been shown to contain viable bacteria after the use of 4% sodium hypochlorite (Siqueira *et al.*, 1997a). Recently, Torabinejad *et al.* (2003a) described a new endodontic irrigant, MTAD, a solution made up of a tetracycline isomer, an acid and a detergent. This irrigant has been shown to efficiently remove the smear layer and it has also been found to be effective at killing *E. faecalis* (Shabahang and Torabinejad, 2003). In line with these findings, a new irrigant, Tetraclean, a mixture of doxycycline, citric acid and polypropylenglycole has been developed. This irrigant was first introduced at the XXV Congress of Italian Society of Endodontics (SIE) in 2004 (Giardino *et al.*, 2004), and the developing research into its actions and features was further described (Giardino *et al.*, 2006; Rimoldi *et al.*, 2006; Giardino *et al.*, 2007).
The aim of this study was to compare the antimicrobial activity of Tetraclean and sodium hypochlorite by *in vitro* and *ex vivo* approaches.

MATERIALS AND METHODS

The methods chosen for the assessment of the bactericidal activity (membrane-filtration for Tetraclean and dilution-neutralization for sodium hypochlorite) are detailed in the European Standard Guidelines for the evaluation of the bactericidal activity of chemical disinfectants (CEN, 1997). In brief, a suspension of *E. faecalis* ATCC 29212 (1.5 to 5 x 10^8 CFU/ml) was incubated with either Tetraclean (kindly provided by Dr. L. Giardino) or sodium hypochlorite (Incofar, Modena, Italy) for 5, 10 or 20 minutes under clean or dirty conditions, i.e. 0.3 g/l Bovin Serum Albumin (BSA - Fluka Biochemika, Germany) or 3 g/l BSA respectively. To assess the efficacy of Tetraclean, after the contact time, each bacterial suspension was filtered with membrane filters (47 mm diameter, 45 µm pore size - Millipore, MA, U.S.A.) to remove the irrigant and to stop its bactericidal action. The membranes were then placed onto Tryptic Soy Agar (TSA - Acumedia, Lansing, MC, U.S.A.) plates and the bacteria were allowed to grow at 37°C for 24 hours. To assess the antibacterial activity of sodium hypochlorite, after the contact time, a neutralizer solution (as detailed in the European Standard Guidelines - CEN, 1997) was added to stop the action of the irrigant. The bacterial suspensions were then placed onto TSA plates and allowed to grow at 37°C for 24 hours. According to the European Standard guidelines, experiments were conducted a) to validate the efficacy of both the membrane-filtration and the dilution-neutralization methods, and b) to rule out any toxicity exerted by the interfering substance, by the neutralizing substance or by the filtration procedure.
For the *ex vivo* study, 75 freshly extracted single-rooted human teeth were used. These teeth were collected from patients of the School of Dentistry of Modena, and they were selected on the basis of their relative dimensions and morphological similarity. 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 (0.85% NaCl). The crowns were flattened using diamond steel discs (Brasseler U.S.A., Savannah, GA) and a final length of 15 mm was achieved for each tooth. Root canal preparation was performed using Niti K3 endodontic instruments (SybronEndo Orange, CA, U.S.A.) 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 and then immersed in saline solution and sterilized by autoclave at 121°C for 30 minutes. After this step they were handled in asepsis under a laminar flow.
In order to evaluate the sterility, 5 teeth, used as negative controls, were incubated at 37°C in sterile Brain Heart Infusion (BHI - Oxoid, Hampshire, U.K.) for 144 hours to rule out any bacterial growth. The remaining 70 out of 75 teeth were infected by immersion for 24 hours in 2 ml of an *E. faecalis* suspension (5 x 10^5 CFU/ml). The efficiency of the method for dentinal tubules infection after a 24-hour period had been established in a preliminary study by scanning electron microscopy. The infected teeth were fixed with formalin and placed in a sterile saline solution and kept at 4°C until they were treated for scanning electron microscopy. S.E.M. samples were obtained using a rotary cutter to create small notches on the external surface of the root; this proce-

dure simplified the following fracture of the root itself that occurred after immersion of the teeth in liquid nitrogen. The fractured samples were then air dried and mounted on stabs prior to be gold-palladium coated and analyzed with the scanning electron microscope.

After 24 hours, 15 out of 70 infected teeth were divided into 3 groups and irrigated (4 ml/tooth rinsing of the root canal followed by 5 minutes immersion in 2 ml/tooth) with sterile BHI broth kept at three different temperatures: 22°C, 37°C and 50°C. These teeth were incubated in fresh BHI broth and used as positive controls. The 55 remaining teeth were divided into 5 groups and each group was irrigated under either of the following conditions:

a) Tetraclean kept at 22°C;
b) Tetraclean kept at 37°C;
c) sodium hypochlorite kept at 22°C;
d) sodium hypochlorite kept at 37°C;
e) sodium hypochlorite kept at 50°C.

As for the positive controls, the irrigation was performed with 4 ml of Tetraclean or sodium hypochlorite into the root canal and by subsequent immersion of each tooth for 5 minutes in the same irrigant solution (2 ml per tooth per tube) maintained at the same temperature used for the injection.

After irrigation, all the teeth were transferred into fresh BHI broth (2 ml/tooth) and incubated at 37°C to verify the effective bacterial removal and the antimicrobial effect of the irrigants by the time. Before the incubation and at time-point intervals for each tooth, 100 µl/tube were collected and plated in duplicate in Slanetz & Bartley Medium (SBM - Oxoid, Hampshire, U.K.) plates. The plates were incubated for 24 hours and then the CFU were counted. This procedure was repeated at time-point intervals of 24 hours for each tooth. The number of CFU detected in the broth was taken as means to assess the microbial load per tooth. Statistical analysis was theen performed.

Statistical analysis was carried out to assess the influence of temperature of irrigation (if any) on the irrigants under investigation. The chi-squared test was used to establish if the temperature exerted any significant effect on the frequency of teeth positive for the presence of bacteria, at time-points after irrigation. Non-parametric tests (Mann-Whitney for Tetraclean and Kruskall-Wallis for sodium hypochlorite) were employed to assess if the temperature played a statistically significant role on the number of CFU/ml, at the same time-points after irrigation. The level of significance was set at 0.05 for all the analyses.

The efficacy (E) of the irrigants was calculated by subtracting to 100 the ratio (expressed as percentage) between the number of positive (P) teeth and the number of irrigated (I) teeth at the end of the experiment:

$$E = 100 - (P/I \times 100)$$

RESULTS

The bactericidal activity of Tetraclean was assessed by means of the membrane-filtration method (CEN, 1997). As shown in Table 1, the CFU in the starting bacterial test suspension matched the amounts required (between 1.5 and 5 x 10^8 CFU/mL). Following different contact times with Tetraclean, few CFUs were detected both under dirty and clean conditions. In all the cases, the reduction of bacteria viability was always greater than 5 logarithms, regardless of the contact times between the bacterial suspensions and Tetraclean, and the interfering substance concentration.

When the bactericidal activity of sodium hypochlorite was assessed by using the dilution-neutralization method (CEN, 1997) no CFU were detected (Table 1), regardless of the conditions tested. According to the European Standard guidelines[12], the validation of both the membrane-filtration and the dilution-neutralization methods was also carried out proving that:

a) no toxic effect was exterted on the bacterial cells by the interferent or the neutralizer;
b) both the procedures were efficient;
c) the only test components with a bactericidal activity were Tetraclean and sodium hypochlorite in each respective test (data not shown).

Once the *in vitro* antibacterial activity of both irrigants has been established, their efficacy was further investigated by means of a previously described *ex-vivo* model of human extracted, decoronated and infected teeth (Shabahang and Torabinejad, 2003; de Almeida-Gomes *et al.*, 2006). In a pilot experiment, the efficiency of the method for the dentinal tubules' infection was established by S.E.M. Figure 1 shows a representative tooth after 24 hours infection. Furthermore, kinetic studies were performed to assess micro-

TABLE 1 - *Bactericidal activity of Tetraclean (as assessed by the membrane-filtration method) and of 5.25% sodium hypochlorite (as assessed with the dilution-neutralization method) against Enterococcus faecalis.*

Irrigants	*Bacterial test suspension (CFU/ml)*	*Interfering conditions*	*Results after different contact times (CFU/ml)* *5 minutes*	*10 minutes*	*20 minutes*
Tetraclean	N = $3.8x10^8$	Dirty conditions (BSA 3 g/l)	10^1 R >10^5	$3x10^1$ R >10^5	$3x10^1$ R >10^5
		Clean conditions BSA (0.3 g/l)	$9x10^1$ R >10^5	$2.2x10^2$ R >10^5	$2.2x10^2$ R >10^5
Sodium hypochlorite	N = $2.1x10^8$	Dirty conditions (BSA 3 g/l)	0 R >10^5	0 R >10^5	0 R >10^5
		Clean conditions (BSA 0.3 g/l)	0 R >10^5	0 R >10^5	0 R >10^5

N = number of bacteria (CFU/ml) in the initial suspensions; R = Reduction in viability.

bial load in the infected and irrigated teeth. As shown in Figure 2, the tooth samples infected 24 hours earlier, always showed comparable levels of CFU (10^9 to 10^{10}).

When the irrigation was performed with BHI broth, a drastic reduction in CFU/ml was observed in all thee teeth (Figure 2a). Following such initial drop, the bacterial load increased again, reaching maximal levels at 24-48 hours after irrigation, regardless of the irrigation temperature used.

Figure 2b shows the effects of irrigation with Tetraclean used at different temperatures. In particular, teeth infected and irrigated with Tetraclean kept at 22°C showed an initial 4 logs drop in CFU that persisted at approximately 5 x 10^5 CFU/ml during the first 72-96 hours, and then dropped to undetectable levels from 120 hours on. Interestingly, when Tetraclean was employed at 37°C, the decrease in microbial load was significantly more rapid, reaching undetectable values (<10 CFU/ml) from 72 hours post-treatment.

Figure 2c shows the results of irrigation with sodium hypochlorite used at different temperatures. The CFU dramatically dropped after irrigation, (8 logs reduction) and the phenomenon

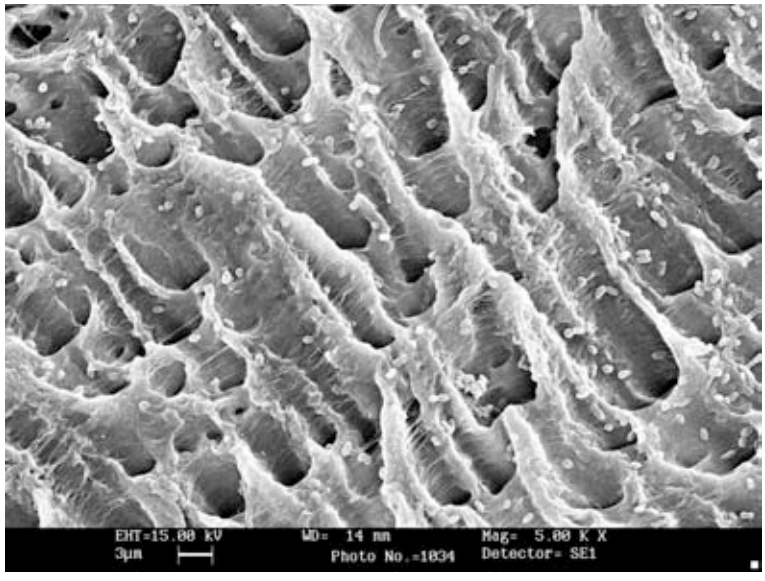

FIGURE 1 - *S.E.M. micrograph of contaminated dentinal tubules after a 24-hour infection period (magnification: 5,000X).*

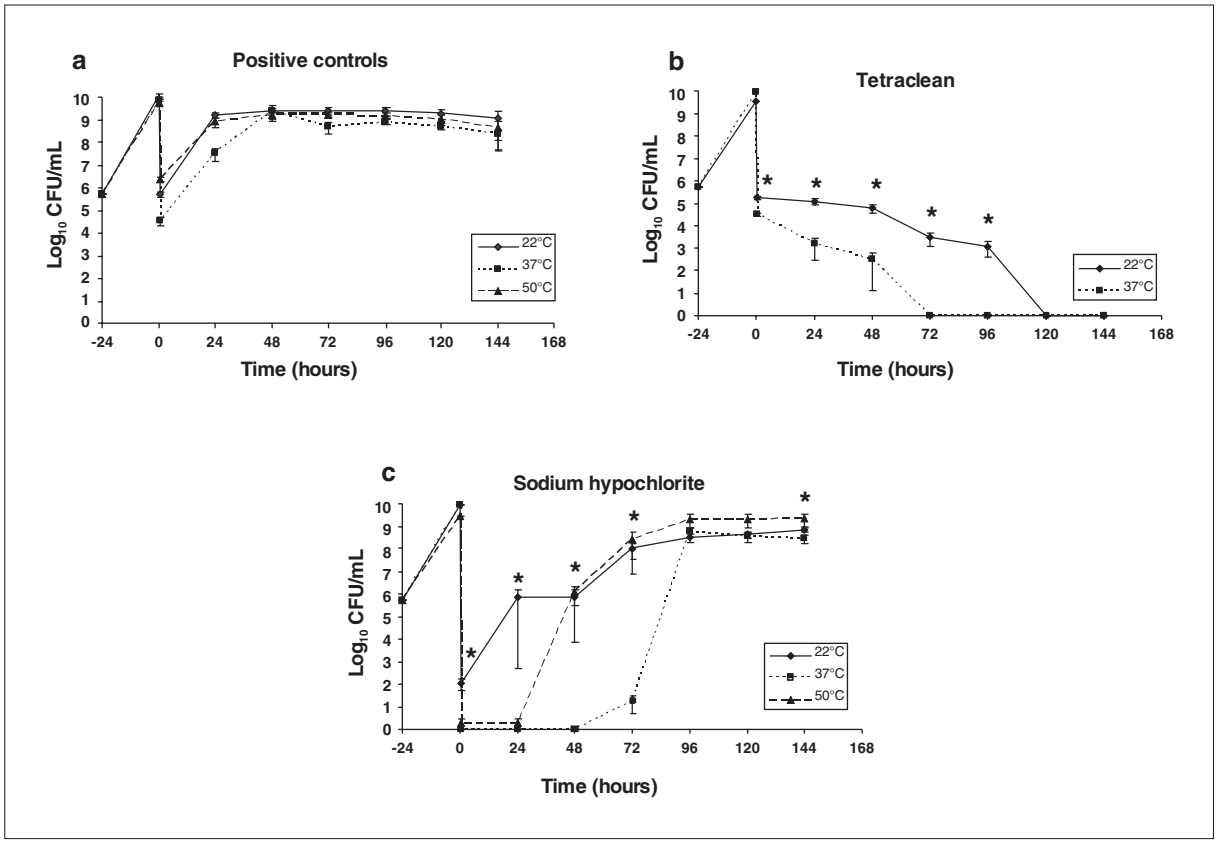

FIGURE 2 - *Kinetics of E. faecalis growth in teeth irrigated with BHI broth (A), Tetraclean (B) and sodium hypochlorite (C). Every curve depicts a temperature of irrigation. The curves were plotted by reporting the mean values of CFU/ml as a function of time. The stars indicate the time points where a statistically significant difference in the number of CFU/ml occurs (as assessed by means of non-parametric tests: Mann-Whitney for Tetraclean; Kruskal-Wallis for sodium hypochlorite). The bars depict the standard error of the mean.*

TABLE 2 - *Influence of the irrigation temperature of Tetraclean and sodium hypochlorite on the bactericidal activity in extracted teeth infected by E. faecalis.*

		Number of positive teeth/Number of irrigated teeth								
Irrigant	*Temperature of treatment*	*Time (hours)*								
		-24 h	*0 h*	*1 h*	*24 h*	*48 h*	*72 h*	*96 h*	*120 h*	*144 h*
Tetraclean	22°C	5/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5	0/5
	37°C	10/10	10/10	10/10	8/10	3/10	0/10	0/10	0/10	0/10
	50°C	Not tested								
	p	n.s.	n.s.	n.s.	n.s.	< 0.05	< 0.001	< 0.001	n.s.	n.s.
Sodium hypochlorite	22°C	15/15	15/15	5/15	7/15	9/15	13/15	14/15	14/15	15/15
	37°C	10/10	10/10	0/10	0/10	0/10	2/10	5/10	7/10	7/10
	50°C	15/15	15/15	1/15	1/15	3/15	11/15	13/15	14/15	15/15
	p	n.s.	n.s.	0.066	<0.05	<0.05	<0.05	<0.05	n.s.	<0.05

The significance p was calculated by using the Chi-squared test. Values lower than 0.05 account for statistically significant differences. n.s. = non significant.

TABLE 3 - *Efficacy of the irrigation with Tetraclean and sodium hypochlorite, carried out at different temperatures in extracted and E. faecalis infected teeth.*

Irrigant	*Temperature of treatment*	*Positive teeth/Irrigated teeth (144 h post-irrigation)*	*Efficacy %*
Tetraclean	22°C	0/5	100%
	37°C	0/10	100%
	50°C	Not tested	
Sodium hypochlorite	22°C	15/15	0%
	37°C	7/10	30%
	50°C	15/15	0%

was particularly evident when the irrigant had been used at 37°C or 50°C (>9 logs reduction). Nevertheless, a bacterial regrowth was observed in most of the sample teeth between 48 and 72 hours post-irrigation, and from 96 hours on, the CFU reached levels as high as the untreated controls, regardless of the irrigation temperature. Once again, the differences among groups were statistically significant in most of the cases, as detailed by the asterisks.

Table 2 provides a summary showing the number of positive teeth and the number of irrigated teeth ratio, at the different conditions, and the corresponding statistical analysis. As shown, the irrigation temperature exerts an influence on the number of positive teeth over the total number of irrigated teeth both by Tetraclean and sodium hypochlorite, the most significant effects being obtained with Tetraclean (37°C, from 72 hours post-irrigation). Table 3 shows the efficacy of the two irrigants 144 hours post-irrigation. The efficacy of Tetraclean was 100%, irrespective of the irrigation temperature used; in contrast, only 30% efficacy was registered when sodium hypochlorite had been used at 37°C, and no effects at all were observed at the other temperatures. As expected, the 5 teeth used as negative controls (uninfected and incubated in sterile BHI throughout the 144 hours of the experiment) did not show any bacterial growth at any time point (data not shown).

DISCUSSION

Several factors, including the complexity of the endodontic anatomy, the persistence of bacteria inside the dentinal tubules (Kakehashi *et al.*, 1965), the occurrence of smear layer and the aggregation of several kinds of bacteria in biofilm, influence a proper disinfection of the RCS during clinical endodontic practice (Siqueira, 2001; Radcliffe *et al.*, 2004). Areas that remain unaffected by the chemomechanical treatment, regardless of the technique and instruments employed, may explain the persistence of microorganisms in the apical portion of the RCS and the consequent endodontic failure (Lin *et al.*, 1991; Siqueira *et al.*, 1997b). The deposition of a heavy smear layer occurring during instrumentation, has been recognized as one of the reasons for the lack of success in endodontic therapy: it may provide a physical shield to bacteria already present in the dentinal tubules, protecting them from irrigants and likely sustaining their growth (McComb and Smith, 1975; Brannstrom, 1984; Torabinejad *et al.*, 2002).

In our studies, *E. faecalis* was chosen as a model microorganism because, amongst all the bacterial species associated with persistent endodontic infections, it is one of the most frequently isolated species (Sundqvist *et al.*, 1998; Hancock *et al.*, 2001; Stuart *et al.*, 2006). *E. faecalis* can be found at depths up to 300 µm within dentinal tubules, where it is able to survive notwithstanding the scant available nutrients, unlike other bacterial species (Portenier *et al.*, 2003). Furthermore, *E. faecalis* appears to be very resistant to the action of endodontic dressing like $Ca(OH)_2$, because of its capability to survive at very high pH (Drucker and Melville, 1971); it can resist heat, U.V., ethanol, hydrogen peroxide and acidity (Portenier *et al.*, 2003); therefore it can persist and survive in treated RCS (Ørstavik and Haapasalo, 1990; Kreft *et al.*, 1992).

Sodium hypochlorite is the most common irrigant employed in endodontic practice. Its antimi-

crobial properties, as well as its toxicity, are proportional to its concentration (Gomes *et al.*, 2001). Several studies have shown that sodium hypochlorite is able to eliminate *E. faecalis in vitro* but it may not be so effective as assessed by *ex vivo* (Siqueira *et al.*, 1997a; Shabahang and Torabinejad, 2003) or *in vivo* studies (O'Hara *et al.*, 1993; Oncag *et al.*, 2003). This led to a demand for novel medicaments capable of higher efficacy against *E. faecalis* and other endodontic pathogens.

In the present paper, Tetraclean and sodium hypochlorite were tested for bactericidal activity *in vitro*. Two different methods (membrane filtration and dilution-neutralization) were employed according to the European Standard Guidelines (CEN, 1997) because of the lack of an effective neutralizer for doxycycline. The results obtained show that both the irrigants are able to cause a CFU reduction (>5 logarithms) such that they can be considered bactericidal. The efficacy of both Tetraclean and sodium hypochlorite is retained under clean and dirty conditions; this implies that their use may be successful also when treating environments known to be contaminated with organic materials, as the infected RCS. In addition, a contact time as short as 5 minutes was sufficient to achieve the impairment required by the European Standard guidelines (CEN, 1997). This finding indicates that, *in vitro*, Tetraclean is at least as effective as sodium hypochlorite in its antibacterial activity.

In further testing the efficacy of both irrigants we chose a well known and already described (Shabahang and Torabinejad, 2003; de Almeida-Gomes *et al.*, 2006) *ex-vivo* model of extracted and experimentally infected human teeth. A 5 minutes irrigation time was chosen as this time point was sufficient to fulfil the required bactericidal effects by both Tetraclean and sodium hypochlorite in the *in vitro* studies. The teeth irrigated with sodium hypochlorite (Figure 2c) showed an immediate and drastic drop (>8 logarithms) in bacterial burden. This decline is likely due partially to the flushing action of the irrigation as also shown by Dametto *et al.* (2005). In addition, a direct antibacterial action of sodium hypochlorite is also likely, according to the recent report by Giardino *et al.* (2007) showing the efficacy of such irrigant on enterococcal biofilm. Nevertheless, a rapid recovery of bacterial growth occurs by 24-48 hours after sodium hypochlorite irrigation in most of the teeth. This finding is in line with a previous report (Shih *et al.*, 1970) showing that bacteria disappear from the RCS immediately after irrigation with 5.25% NaOCl and re-colonize the root canals in 80% of specimens after 2-7 days (Oliveira *et al.*, 2007). The bactericidal activity of sodium hypochlorite in the RCS irrigation may be just superficial because of its inability to remove the smear layer and its weak penetration into dentinal tubules (Berutti *et al.*, 1997). These experimental data may explain the clinical observations on the doubtful efficacy of sodium hypochlorite in intracanalar treatment (Oncag *et al.*, 2003), unless it is used in combination with chlorexidine (Hancock *et al.*, 2001). When compared to sodium hypochlorite, Tetraclean is less effective in reducing the microbial load immediatly after irrigation. Interestingly, the number of bacteria in teeth treated with Tetraclean dropped gradually and it reached zero in 100% of the cases, by 72 hours or 120 hours for teeth irrigated at 37°C or 22°C, respectively. This trend can be due to the properties of the single components of this mixture. Firstly, the smear layer that covers the root canal surface and sometimes plugs the entrance to the dentinal tubules can be effectively removed by citric acid; therefore, areas previously inaccessible can be reached by the irrigant. Secondly, the low surface tension of Tetraclean (Giardino *et al.*, 2006) may favor the deep penetration into the tubules by increasing the wetting of the dentinal walls. Thirdly the doxycycline exerts a gradual and prolonged bactericidal effect on the bacterial cells (Stabholz *et al.*, 1993) because of its well known binding capability to dentine. This would ultimately result in long-lasting release of active molecules. In addition it has been reported that *E. faecalis* is resistant to a wide spectrum of antibiotics, while displaying some degree of susceptibility to tetracyclines (Tripodi *et al.*, 1995; Savoia *et al.*, 1996). Finally, the temperature also adds up to the action of Tetraclean, as shown by the fact that the irrigation carried out at 37°C provides bactericidal effects in a higher number of teeth and in a shorter time with respect to the irrigation carried out at 22°C (Figure 2b and Table 2). This same effect is evident, though to a lesser extent, for the teeth irrigated with sodium hypochlorite, while the 50°C irrigation was not

performed with Tetraclean, being doxycycline thermolabile.

It is worth noting that Tetraclean is similar to MTAD, a mixture described by Torabinejad and coworkers (Torabinejad *et al.*, 2003a; Torabinejad and Johnson, 2003) that is known to be effective in:

a. removing the endodontic smear layer (Torabinejad *et al.*, 2003b);
b. eliminating microbes that resist conventional endodontic irrigants and dressings (Shabahang and Torabinejad, 2003);
c. providing local antimicrobial activity through the affinity of doxycycline for dental tissues (Baker *et al.*, 1983). The two main differences between Tetraclean and MTAD consist in the different detergent and in the threefold less content of doxycycline in Tetraclean.

Overall, two major conclusions may be drawn:

a) the *in vitro* antimicrobial activity of Tetraclean, which is rapidly fulfilled within 5 minutes;
b) the *ex vivo* efficacy observed in infected human teeth, where the RCS irrigation made by this mixture allows successful (100% efficacy) microbial clearance.

Notwithstanding the low number of teeth tested and the limits of the *ex vivo* model, our data make us confident that further studies will establish the efficacy of Tetraclean against other bacterial endodontal pathogens and its real potential in daily clinical practice.

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