〔Original〕

Application of tea tree oil for disinfection of dental unit waterlines

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Abstract

Purpose : The purpose of this study was to verify the efficacy of tea tree oil (TTO) derived from *Melaleuca alternifolia* for the disinfection of dental unit waterlines. Materials and Methods : Twenty-six microorganisms were isolated from the water samples taken from dental unit waterlines. TTO 2.0% solubilized with phosphate buffered saline (PBS) containing Tween 20 was used as a disinfecting reagent. The effect of the disinfecting reagent was determined through analysis of the microorganisms isolated from the water samples and the biofilm that formed on the inner surface of the warmer tank. The same analysis was applied directly to the dental unit waterlines. Results : The microorganisms isolated from the water samples and biofilm on the inner surface of the dental unit warmer tank were completely inhibited by use of a disinfecting reagent containing 2.0% TTO. Additionally, no microorganisms were detected in the water after direct application of the disinfecting reagent to the dental unit waterlines, although the mean (SE) of the viable microorganism count was 2.43 (0.07) CFU/mL ($\times 10^4$) before application. Conclusion : We conclude that the use of TTO as a disinfecting reagent for dental unit waterlines would be an efficient way to achieve complete disinfection of the dental unit waterlines and ensure the safety of the human oral cavity.

INTRODUCTION

The essential oil of *Melaleuca alternifolia*, also known as tea tree oil (TTO), has been used for medicinal purposes in Australia for more than 80 years (Carson et al., 1993). The tree itself has been used therapeutically for a significant period of time and is a component of the traditional medicine of the Bundjalung aborigines of northern New South Wales (Carson et al., 1993). The essential oil is obtained by steam distillation and contains approximately 100 components, which are mostly monoterpenes (Brophy et al., 1989). The components of commercial TTOs must fall within the percentage composition ranges stipulated in the International Standard 4730 for "Oil of *Melaleuca*, terpinen-4-ol type" (ISO, 1996).

TTO and several of its components exhibit broad-spectrum antimicrobial (Carson et al., 1993) and anti-inflammatory (Brand et al., 2001 ; Koh et al., 2002) activities *in vitro*. These properties have prompted its use in the treatment of a range of superficial conditions, including cuts, insect bites, boils, acne, and tinea (Carson et al., 1993 ; Bassett et al., 1990). Furthermore, data from recent clinical studies indicate that superficial infections or conditions caused by bacteria (Bassett et al., 1990), fungi (Jandourek et al., 1998 ; Vazquez et al., 2002) and viruses (Carson et al., 2001) show clinical responses to treatment with TTO. Anecdotal and scientific evidence also suggest that TTO may be useful in the maintenance of oral hygiene and prevention of dental disease (Groppo et al., 2002 ; Shapiro et al., 1994 ; Walsh et al., 1987). Furthermore, oral bacteria show susceptibility to TTO *in vitro* (Hammer et al., 1999).

Dental unit waterlines are contaminated with numerous microorganisms derived from biofilms that form on the inner surface of the waterlines (Barbeau et al.,

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1996 ; Williams et al., 1996). It has also been shown that the contaminating microorganisms in dental unit waterlines can pose a serious threat of infection to dental patients. Furthermore, Araki et al. (Araki et al., 2000) reported the presence of many viable microorganisms in dental unit waterlines, including *Legionella*, *Streptococcus*, *Escherichia*, *Sphingomonas*, *Methylobacterium*, and *Pseudomonas* (Araki et al., 2000). The presence of *Staphylococcus*, *Mycobacterium*, *Candida*, and other types of microorganisms in dental unit waterlines has also been reported (Pankhurst, 2003 ; Walker et al., 2007 ; Williams et al., 1996). Additionally, further studies have demonstrated that these microorganisms could become opportunistic pathogens (Abe, 1988, Luigi et al., 2010, Ronald et al., 1995 ; Walker et al., 2004). Moreover, exposure to highly contaminated water may have detrimental effects on immunocompromised patients (Jorgensen et al., 1999 ; Szymańska, 2000).

Although flushing with city water has been shown to cleanse contaminated dental unit waterlines, even time-dependent flushing is minimally effective in eliminating microorganisms (Charles et al., 2002). To avoid contraction of infections from dental unit waterlines, many chemical reagents, such as chlorhexidine, hydrogen peroxide, and chlorine dioxide, have been utilized (Walker et al., 2007). Furthermore, acidic electrolyzed water has been used for the treatment of dental unit waterlines (Kohno et al., 2004). Even though some chemical reagents can eliminate the contaminating microorganisms in waterlines, residual reagents in the waterlines may cause toxicity and acridity for the cells of the human oral cavity (Miller et al., 2000). Therefore, the development of a safe, nontoxic, disinfecting reagent suitable for use in dental unit waterlines is required.

Till date, essential oils have not been used as reagents for the disinfection of dental unit waterlines. However, given the antimicrobial qualities of TTO described above, it may be an appropriate agent for treating and disinfecting dental unit waterlines. Additionally, no organisms have been reported to be resistant to TTO, despite its medicinal use in Australia since the 1920s (Carson et al., 2006). Furthermore, TTO has shown wound healing and immunostimulatory activities in patients receiving dental treatment (Carson et al., 2006). Therefore, the purpose of this study was to verify the efficacy and suitability of TTO as a disinfecting reagent for dental unit waterlines.

MATERIALS and METHODS

Tea tree oil

TTO was purchased from E-NESS CO. Ltd., Yokohama, Kanagawa, Japan. The oil composition was determined as previously described (Hammer et al., 1999) using gas chromatography-mass spectrometry, which was performed at the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia. The levels of terpinen-4-ol and 1,8-cineole were 41.5% and 2.1%, respectively, in compliance with the International Standard 47320 (ISO, 1996).

Collection of water from dental unit waterlines

We selected a single dental unit in the general dentistry clinic of the Health Sciences University of Hokkaido. To determine the contaminating microorganisms in the dental unit waterlines, we collected the following 3 types of water samples using a 3-way syringe that fed into a sterilized tube (5 mL) : (1) One Night Water, the water in the dental unit that was not used for 1 night ; (2) Two Day Water, the water in the dental unit that was not used for 2 days (i.e., a weekend) ; and (3) One Week Water, the water in the dental unit that was not used for 1 week (i.e., a long vacation).

Culture of bacteria in the water

Each collected water sample was dispersed and diluted with 10 mM phosphate-buffered saline (PBS). A small volume (100 μL) of the sample was inoculated on a peptone-yeast extract glucose (PYG) agar plate, and the microorganisms in the water were cultured. The culture conditions are reported in a previous study (Hosaka et al., 2001). After culture, the colonies on the agar plates were counted and examined with respect to the shape, color, size, and Gram staining. Finally, those microorganisms showing different colony shapes were isolated from the agar plates and cultured individually for subsequent experiments in order to examine the disinfection ability of TTO.

Examination of flushing effects

The effects of flushing for 1 min and 5 min were ex-

amined for the dental unit after it remained unused for 2 days. To determine the effects of flushing, 3 dental units were selected from the general dentistry clinic of the Health Sciences University of Hokkaido. Firstly, Two-day Water samples were collected from each dental unit using 3-way syringes. After flushing for 1 min and 5 min, water samples were collected again. Subsequently, the microorganisms in these water samples were cultured to estimate the total number of microorganisms.

Preparation of TTO

Tween 20 was used as a solubilizing solution, and TTO was solubilized to 0.7% or 2.0% in phosphate buffered saline (PBS) containing 0.6% Tween 20 and used as a disinfecting reagent.

Examination of the disinfection of cultured microorganisms

Viable microbial cells were obtained in a pure culture after isolation from each of the 3 water sample types. These cells were washed with PBS and subsequently treated with the disinfecting reagent containing TTO in an Eppendorf tube (10^7 cells/mL) for 5 min or 10 min at room temperature. The microbial cells were then washed, centrifuged, and suspended in PBS. After a 10-fold serial dilution, 100 μL of each microbial sample was inoculated on a PYG agar plate and incubated for 7 days. The total number of microorganisms grown on the agar plate was counted and estimated as the colony-forming unit (CFU).

Examination of the disinfection of the biofilm on the inner surface of the warmer tank

The warmer tanks supplying the dental unit waterlines were cut open, and pieces of biofilm debris from the inner surfaces of the tanks (Figure 1) were collected using a sterilized micro-spatula. The pieces of debris (each lump weighing approximately 30 mg) were immediately soaked in the disinfecting reagent containing 2.0% TTO in an Eppendorf tube for 10 min at room temperature. The pieces were then homogenized, washed with PBS, centrifuged, and suspended in fresh PBS. After a 10-fold serial dilution, 100 μL of each solution was inoculated on a PYG agar plate and incubated for 7 days. The total number of microorganisms grown on the agar plate was subsequently counted and estimated as CFUs.

Application of the disinfecting reagent to the dental unit waterlines

The disinfecting reagent was directly applied to the dental unit waterline using a cleaning system (Figure 2). This system was a trial model produced by Morita Corporation, Saitama-shi, Saitama, Japan.

Before cleaning with the disinfecting reagent, Two Day Water and One Week Water samples were collected using a 3-way syringe. The dental unit waterline was then filled with the disinfecting reagent via the cleaning system and maintained for 10 min. Finally, city water was allowed to flow through the cleaning system to remove any residual reagent in the dental unit waterline. Post-cleaning, an initial water sample was collected using the 3-way syringe. To estimate the disinfecting effect of TTO, the microorganisms in these water samples were cultured on agar plates and the number of CFUs determined.

Statistical analysis

The experiments were performed in triplicate and the means and standard error calculated. Statistical significance was determined using repeated measures analysis of variance (ANOVA) with ystat 2008 software. A p-value <0.05, <0.01, or <0.001 were considered statistically significant.

RESULTS

Examination of the CFUs in the 3 types of water samples (One Night Water, Two Day Water, and One Week Water) showed that the longer the dental unit re-

Fig.1 Inner surface of the warmer tank of a dental unit

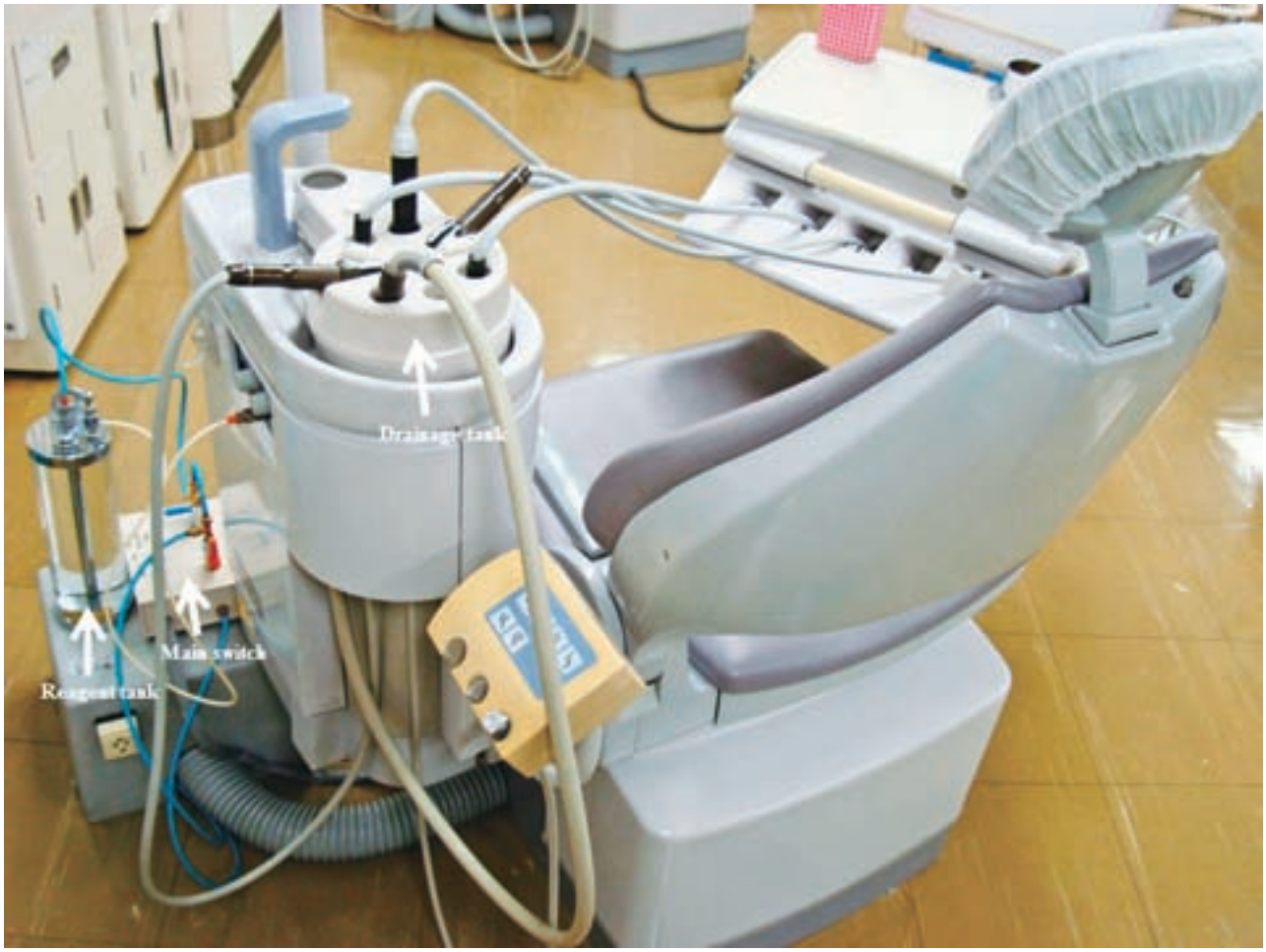

Fig.2 Dental unit waterline cleaning system(trial mode)

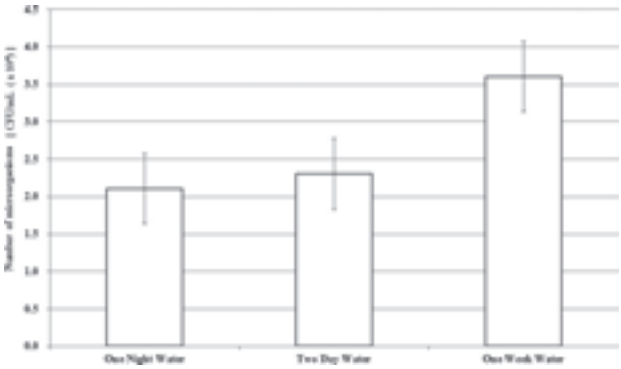

Fig.3 The number of microorganisms in One Night Water, Two Day Water, and One Week Water

mained unused, the more contaminated the dental unit waterline became (Figure 3). The mean (SE) of the viable microorganism count was 2.27 (0.09) CFU/mL (× 10^4) in the One Night Water, 2.43 (0.07) CFU/mL (× 10^4) in the Two Day Water, and 3.63 (0.07) CFU/mL (× 10^4) in the One Week Water.

For Two-day Water, flushing for 1 min and 5 min slightly reduced the number of microorganisms in the 3 dental unit waterlines (Figure 4). The mean (SE) viable microorganism count in the 3 lines was 2.80 (0.34) CFU /mL (× 10^4) before flushing, 2.30 (0.30) CFU/mL (× 10^4) after 1 min of flushing, and 1.60 (0.25) CFU/mL (× 10^4) after 5 min of flushing ; however, after application of the 2.0% TTO disinfecting reagent, the microbial counts were 0 CFU/mL in all 3 lines (Figure 4).

In the present study, 26 types of microorganism (from A to Z in Figure 5) were isolated from the 3 water samples. These microorganisms were cultured and treated with a disinfecting reagent containing TTO.

Of the 26 microorganisms isolated from the dental unit waterlines, 13 isolates were inhibited (0 CFU/mL) after treatment with the disinfecting reagent containing 0.7% TTO for 5 min (Figure 5). Twelve isolates (B, D, G, M, O, P, Q, R, S, T, V, and W) were inhibited (0 CFU/ml) by treatment with a 2.0% TTO disinfecting reagent for 5 min (Figure 5). Finally, the M isolate was completely inhibited after treatment with the 2.0% TTO disinfecting reagent for 10 min (data not shown). On the other hand, it was demonstrated that 0.6% Tween 20,

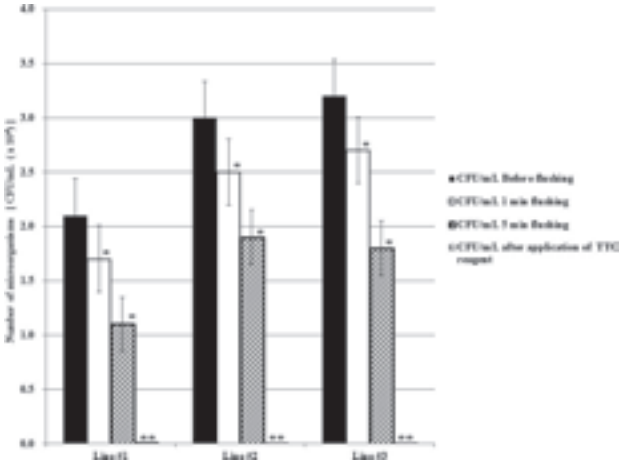

Fig.4 The number of microorganisms before and after flushing and after application of a 2.0% TTO disinfecting reagent
*P<0.05 against the value before flushing.
**P<0.001 against the value before flushing.

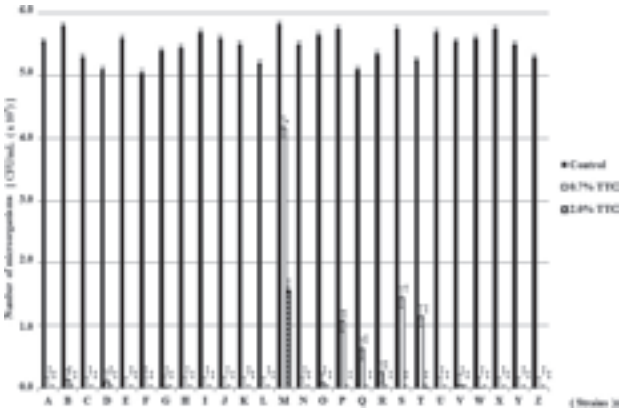

Fig.5 Sterilizing effects on 26 microorganisms isolated from the dental unit waterlines.
*P<0.05, †P<0.01, ‡P<0.001 against the control.

which was used as the solubilization solution, did not inhibit the microorganisms (Anjali et al., 2010).

In the biofilm model, flakes from the biofilm that had formed inside the warmer tank of the dental unit, were treated with the 2.0% TTO disinfecting reagent. Following this, the pieces of biofilm debris were washed and suspended in PBS, then inoculated on the agar plates. Subsequently, no colony formation was detected on the agar plates after 7 days (data not shown). On the other hand, many colonies, ranging from 1.3×10^6 CFU/mL to 3×10^7 CFU/mL, were detected on the agar plates inoculated with the untreated biofilm flakes. These results indicate that 2.0% TTO is an effective disinfecting reagent for the eradication of biofilm-forming microorganisms.

Additionally, the 2.0% TTO disinfecting reagent was applied directly to the waterlines of the dental units that had not been used for 2 days. Consequently, no microorganisms were detected in the water obtained from these dental unit waterlines (Figure 4).

DISCUSSION

Consistent with previous reports, the present study shows that dental unit waterlines have high levels of microbial contamination. This observation appears to be nearly universal, regardless of whether the dental units are connected to municipal water supplies or equipped with a separate water system (Molinari, 1994 ; Shearer, 1996).

This study also demonstrates that flushing the waterlines for 1 min or 5 min is not effective in reducing the overall number of microorganisms in the dental unit waterlines. This result supports the findings of Charles et al. (Charles et al., 2002).

Previously it was reported that many microorganisms such as *Legionella*, *Streptococcus*, *Pseudomonas*, *Escherichia*, *Sphingomonas*, and *Methylobacterium* (Araki et al., 2000) as well as *Staphylococcus* and *Candida* (Pankhurst, 2003 ; Williams et al., 1996 ; Walker et al., 2000) were also detected in dental unit waterlines. In the present study, 26 types of microorganism, showing various colonizers, were isolated from the 3 water samples (One Night Water, Two Day Water, and One Week Water), although these isolates were not identified at the genus/species level. Based on previous data, it was assumed that the 26 microorganisms isolated in this study were closely related to previously reported microorganisms.

Generally, microorganisms found in dental unit waterlines are known to be nonpathogenic, but it is frequently reported that these microorganisms may cause opportunistic infections (Abe, 1988 ; Luigi et al., 2010 ; Ronald et al., 1995 ; Walker et al., 2004). In addition, dentists, who are exposed to a high microorganism load on a daily basis, are at increased occupational risk of contracting infections. Several studies have reported high rates of respiratory infections in dentists and dental personnel (Davies et al., 1994 ; Mikitka et al., 1995), and at least 1 dentist has died after being infected with *Legionella* from a dental unit (Ronald et al., 1995). In the present study, 50% of the 26 microorganisms isolated from the dental unit waterlines were inhibited by a dis-

infecting reagent containing 0.7% TTO, while the remaining 50% were inhibited completely by a 2.0% TTO disinfecting reagent (Figure 5).

It is well known that microorganisms form biofilms on the inner surface of many types of pipelines, and these microorganisms, shielded by the biofilm, are highly resistant to various antimicrobial agents, immunocytes, and antibodies (Høiby et al., 2011). Therefore, it is assumed that microorganisms detected in dental unit waterlines also form biofilms. In particular, microorganisms located inside the warmer tank of a dental unit are exposed to ideal environmental conditions that result in biofilm formation. To examine the effect of TTO on biofilm-forming microorganisms, pieces of biofilm debris obtained from the inner surface of the warmer tank were soaked directly in a disinfecting reagent containing 2.0% TTO, and no microorganisms were detected in the debris after treatment. This result demonstrates that the disinfecting reagent containing 2.0% TTO can be used to effectively inhibit the biofilm-forming microorganisms obtained from the inner surface of the warmer tank of a dental unit.

The results of this study demonstrate that no microorganisms were detected in the water samples obtained from the dental unit waterlines after cleaning with the reagent containing 2.0% TTO (Figure 4 : after application of TTO reagent). These results demonstrate that TTO at a concentration of 2.0% is effective for disinfecting dental unit waterlines.

In the present study, TTO was solubilized with PBS containing 0.6% Tween 20 and used as the disinfecting reagent. When the 26 microorganisms isolated from the dental unit waterlines were treated with PBS containing 0.6% Tween-20 without TTO, no disinfecting effects were observed. These results indicate that the disinfecting activity of the reagent was directly attributable to the 2.0% TTO. These results also demonstrate that TTO is very effective against a variety of microorganisms and can be used for the disinfection of dental unit waterlines.

Moreover, it was confirmed that TTO exhibits strong antibacterial effects against almost all bacteria (including methicillin-resistant *S. aureus*), viruses (including herpes simplex virus type 1 and type 2), and fungi as previously reported by Carson et al. (Carson et al., 2006). In recent years, it has been reported that the hepatitis B virus (HBV) can invade dental handpieces used on patients with HBV infection (Miller et al., 2000). Many recent studies have indicated that TTO is effective against enveloped and nonenveloped viruses (Carson et al., 2006). These results suggest that TTO may eliminate many types of bacteria, fungi, and viruses (including HBV) from dental unit waterlines. Furthermore, TTO also has positive effects on wound healing and immunity (Carson et al., 2006), and therefore, can have secondary benefits to patients. Moreover, TTO is thought to be environmentally friendly and inexpensive, because only a small amount of product is required. Given our findings, we conclude that using TTO as a disinfecting fluid for dental unit waterlines is an efficient method of maintaining complete sterility of these units and ensuring protection of the human oral cavity.

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