Brief Report

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A pilot study comparing the disinfecting effects of commercialized stable ClO_2 solution (free of activation) with conventional H_2O_2 on dental unit waterlines in the dental practice setting

Xiaolei Zhang[1,§], Jingjing Sha[2,§], Zefan Huang[3], Sisi Chen[2], Xufei Luo[1], Ruijun Liu[4], Tetsuya Asakawa[5,*], Qiang Zhang[6,*]

[1] Department of Hospital Infection, Shenzhen Stomatology Hospital, Shenzhen, China;
[2] Department of Endodontics, Shenzhen Stomatology Hospital, Shenzhen, China;
[3] Department of Nursing, Shenzhen Stomatology Hospital, Shenzhen, China;
[4] Department of Periodontology, Shenzhen Stomatology Hospital, Shenzhen, China;
[5] Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, Shenzhen, China;
[6] Department of Orthodontics, Shenzhen Stomatology Hospital, Shenzhen, China.

SUMMARY Disinfection of dental unit waterlines (DUWLs) plays a key role in control and prevention of nosocomial infection in a dental clinic. The most conventional disinfectant is hydrogen peroxide (H_2O_2), while chlorine dioxide (ClO_2) has been considered however was limited by the "activation" procedures. With the availability of commercialized stable ClO_2 solution (free of activation), direct application of ClO_2 in the dental practice became possible. This study was designed to compare the disinfecting effects of stable 5 ppm of ClO_2 solution with conventional 0.24% of H_2O_2 on DUWLs in dental practice. Studies of colony-forming units (CFUs), confocal laser scanning microscopy (CLSM) and scanning electron microscope (SEM) were employed for evaluation. In CFUs studies, we found that the efficiency of ClO_2 was no less than those of H_2O_2. In the morphological studies, the stronger disinfecting effects of ClO_2 was verified by both CLSM and SEM studies for removal and prevention of biofilm. Importantly, ClO_2 solution achieved a better disinfecting efficiency not only at the surface of bacterial biofilm, but also, it has penetrating effects, presented disinfecting effects from the surface to the bottom of the biofilm. This pilot study provided evidence regarding the efficiency of stable ClO_2 solution on disinfection of DUWLs in the dental practice setting. Application of stable ClO_2 solution in dental practice is therefore become possible.

Keywords dental unit waterlines, hydrogen peroxide (H_2O_2), chlorine dioxide (ClO_2), disinfection, biofilm

1. Introduction

Dental unit waterlines (DUWLs) are a piping system providing pure water for dental treatment. This system is comprised of several narrow, long pipelines, which is often intermittent used with unbalanced and slow flows. Accordingly, DUWLs are easily contaminated by bacteria and then induced bacterial biofilm formation. Frequently used positions, such as air/water syringe, dental hand piece, and cuspidor faucet usually have more chance to be contaminated, potentially conduct bacteria to the waterline, and promote biofilm formation (*1*). It has been documented that bacterial biofilm on DUWLs is widely distributed, with the approximately 30-50 μm thickness, which is believed to potentially cause serious waterline contamination (*2*). If such contamination is neglected, the floating microorganisms or dissociative biofilms might be transferred to the patient, or come to the air through a handpiece, thereby increasing the infectious risks to patients and dental staffs (*3*). Hence, surveillance and prevention of DUWLs-related contamination are routine works of a dental clinic. A battery of disinfectants and disinfecting methods, such as hydrogen peroxide (H_2O_2) (*4*), chlorine dioxide (ClO_2) (*5*), chlorhexidine gluconate (*6*), sodium hypochlorite (*7*), peracetic acid (*8*), intermittent sterilization with peracetic acid/H_2O_2 (*9*), continuous disinfection with hydrogen peroxide/silver ions (*6*) were investigated for use in DUWLs. Nonetheless, only few of them are actually used in a dental clinical setting for various reasons. An ideal disinfectant for using in the dental practice setting should have several characteristics, such as effective, safe, appropriate priced, convenient, and easily available. Accordingly, H_2O_2 is the most

commonly used disinfectant for DUWLs clinically.

ClO_2 is an effective, safe high-level disinfectant, which is widely used for disinfection of environments, surface of articles, and human. It has been reported using ClO_2 for oral cleaning (*10-12*) and wound cleaning (*13*). However, ClO_2 is not commonly used for dental clinical setting because it is difficult to obtain a stable ClO_2 solution, and store it for a long time. Hence, the ClO_2 solution usually has to be prepared before using by a chemical reaction of precursors, which is termed as "activation", that is inconvenient and unsafe for DUWLs in the actual clinical setting (*14*) because the reaction concentration is not easily controlled. Our previous studies mentioned availability of a commercialized stable ClO_2 solution that was free of activation (*14,15*), that make it possible for convenient use of ClO_2 in clinical setting since we can purchase the stable solution with a certain concentration. On the other hand, colony-forming units (CFUs) have been used as a standard index for evaluating the efficiency of disinfection in DUWLs scenario. Conversely, remove/control of bacterial biofilm during the disinfection in DUWLs has never been a standard index, even though it plays a key role in prevention and intervention of the DUWLs contamination.

Based on the aforementioned contexts, we designed this pilot study to compare the efficiency of disinfection in DUWLs between the conventional H_2O_2 and the commercialized stable ClO_2 solution (free of activation) in the clinical practice. Meanwhile, we also attempted to observe the changes of bacterial biofilm along with the CFUs affected by ClO_2 solution and H_2O_2. We believe that the findings of this study will be useful for better understanding the efficiency of the commercialized stable ClO_2 solution (free of activation) as well as changes of bacterial biofilm affected by ClO_2 and H_2O_2, that is useful for selection of an appropriate disinfectant for DUWLs in the dental practice setting.

2. Materials and Methods

2.1. Preparation of DUWLs and collection of the water samples

Experimental DUWLs in the present study were derived from the dental chair units (DCUs, UTTG27959, Planmeca, Helsinki, Finland), which had been normally used for the routine clinical practice for three years. Total 18 DCUs were involved in this study, where 12 DCUs were allocated to the ClO_2 group and 6 were allocated to the H_2O_2 group using a simple coin toss randomized method. Two sorts of disinfectants were prepared in the present study, namely 5 ppm of commercialized stable ClO_2 solution (free of activation) which was purchased from the manufacturer (Shenzhen Caseche Biotech Co., Ltd., Shenzhen, Guangdong, China) and 0.24% H_2O_2 (*4*). Concentrations of the agents were determined according to the previous studies using ClO_2 (*16,17*) and H_2O_2 (*4*) for disinfection.

Once the investigation initiated, 500 mL ClO_2 and H_2O_2 solutions were put into the sterilizing bottle of DCUs respectively after the daily dental clinical work was finished. The disinfection procedures were opened for 4 min (wash with disinfectant for 2 min and then wash with pure water for 2 min); then the power switch was turned off overnight. Water samples were collected before the clinic work at the next morning. Sampling was performed as per the 2023 Guidelines for Infection Control and Management in Dental Unit Waterlines (*18*). Sampling was implemented at three positions, namely air/water syringe, dental hand piece, and cuspidor faucet following the principles of aseptic operation. Experiments were performed for 45 weeks, except the previous day for the baseline test. Water samples were measured once per week for the first 29 weeks, and once per two weeks for the last 16 weeks.

2.2. Detection of the CFUs in water samples

In terms of CFUs test, 200 μL sample water was put into a sterile petri dish, and mixed with medium, subsequently cultured at 37°C for 48 hours. CFUs were calculated as the numbers of bacterial colonies divided by the volume of diluent. Less than 100 CFUs/mL is considered as negative.

2.3. Confocal laser scanning microscopy (CLSM) study

After 12 weeks of disinfection (ClO_2 or H_2O_2), Waterline samples of DUWLs were cut into rings (0.2-0.5 mm length), which were immediately exposed to a LIVE/DEAD BacLight Bacterial Viability Kit (Cat. No. L7012, Thermo Fisher Scientific Inc., Waltham, MA, America) for 10 min, washed with PBS for 1 min, and then rinsed twice. Non-invasive CLSM images were acquired on the complete biofilm at the inner wall of DUWLs using a CLSM (FV3000, Olympus, Tokyo, Japan) (excitation light wavelength = 510/480 nm). Vital fluorescence staining (VFS) was performed as per the manufacturer's manual. Bright green staining displays live bacteria, red staining shows dead bacteria, and the yellow staining is the overlap (coexistence) of dead and live bacteria.

Image analysis was performed using an ImageJ 1.34p software (National Institutes of Health, Bethesda, MD, USA; *http://rsb.info.nih.gov/ij/*). Images of each color channel were assembled into stacked images, and the areas occupied by live bacteria and dead bacteria were calculated respectively. The ratio of live bacteria to dead bacteria was calculated and submitted to statistical analysis.

2.4. Scanning electron microscope (SEM)

Remaining waterline samples of DUWLs undergone

a 12-week sterilization were cut into a 1 cm section, then cut vertically from the middle line. All samples were placed into 2.5% glutaraldehyde for overnight fixation. After being dehydrated by ethanol gradient (30%, 50%, 70%, 80%, 85%, 90%, 95%, and anhydrous ethanol for 0.5 h at each concentration), the tubes were fixed on a special aluminum base. After spraying gold nanoparticles, they were observed and photographed using a scanning electron microscope (Su8220, Hitachi, Tokyo, Japan).

2.5. Statistics

A SPSS soft (V26.0.0, IBM, Armonk, NY, USA) was used for statistical analyzes. Comparisons of proportion were performed with a Chi-square test. The quantitative VFS data were compared using a Mann-Whitney *U* test. $p < 0.05$ was considered as the statistical significance.

3. Results and Discussion

In the present study, we compared the disinfecting effects of commercialized stable ClO_2 solution (free of activation) with conventional H_2O_2 for DUWLs by observing the states of biofilm. Our data suggest a better disinfecting efficiency of this ClO_2 solution than that of conventional H_2O_2 in terms of DUWLs disinfection. To the best of our knowledge, this study is the first study to evaluate the efficiency of commercialized stable ClO_2 solution (free of activation) using in disinfection of DUWLs. We believe that the findings of this study are helpful to select an appropriate disinfectant for DUWLs in the routine dental practice.

3.1. Analysis of the CFUs in the DUWLs

As shown as in Table 1, total 1,998 water samples were tested, of those, 1,332 were in ClO_2 group, and 666 were in H_2O_2 group. In the ClO_2 group, total 1,312 samples were identified as "-" once their detection values < 100 CFU/mL, the pass rate was 98.48%. In the H_2O_2 group, total 648 samples were identified as "-", the pass rate was 97.30%. No significant difference was found between groups in total ($\chi^2 = 3.434$, $p = 0.064$). In terms of different positions, no signification difference was found between two groups (Table 1). These data indicated that the disinfecting efficiency of this stable ClO_2 solution was no weaker than those of conventional H_2O_2

Table 1. Analysis of the colony-forming units in the dental unit waterlines

Positions	ClO_2 -/+	H_2O_2 -/+	X^2	*p* value
Air/water syringe	440/4	220/2	0	1.000
Dental hand piece	440/4	216/6	3.249	0.071
Cuspidor faucet	432/12	212/10	1.504	0.220
Total	1312/20	648/18	3.434	0.064

"-" means the detection value < 100 CFUs/mL; "+" means the detection value ≥ 100 CFUs/mL, CFUs = colony-forming units.

3.2. Comparison of the disinfecting effects between ClO_2 and H_2O_2 by observing the changes of the biofilms

As shown as in Figure 1, the disinfecting effects between ClO_2 and H_2O_2 were compared with a CLSM along with a SEM. Biofilm is a thin layer at the surface of waterline. The results of the CLSM displayed that multitudes bacteria in the biofilm were

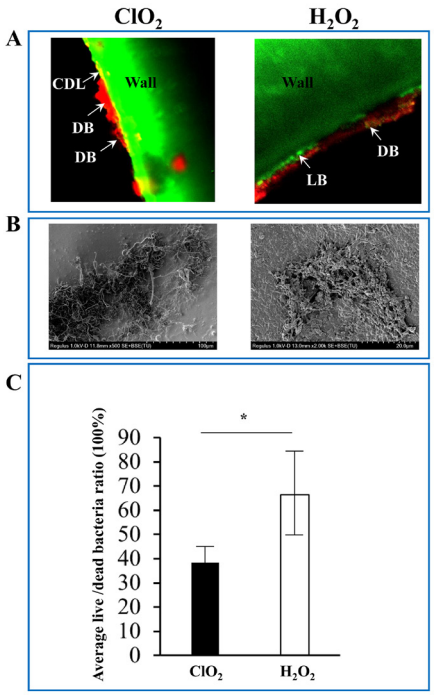

Figure 1. Comparison of the disinfecting effects between ClO_2 and H_2O_2 by observation of the changes of biofilms. A. Representative images of CLSM displaying the disinfecting effects on the biofilm (ClO_2 *vs.* H_2O_2). Green background is the wall of the waterline ("Wall" in the figure). Biofilm is a thin layer at the surface of waterline, where bright green patches represent live bacteria ("LB" in the figure); red patches represent dead bacteria ("DB" in the figure); yellow patches represent coexistence dead and living bacteria ("CDL" in the figure). In the ClO_2 group (left column), bright green patches could not be observed, the intermittent biofilm included large red patches and underneath linear yellow stripes indicating bacteria in the biofilm were disinfected. Moreover, ClO_2 had effects of infiltrating into the biofilm (penetrating effects). Whereas in the H_2O_2 group (right column), bright green patches were still visible indicating the survival of numerous live bacteria indicating the disinfecting effects were not satisfactory. No more penetrating effects were found here, hence, H_2O_2 exhibited a modest effect on killing the bacteria at the bottom of the biofilm. These data suggested a better efficacy of ClO_2 in removal of biofilm. **B**. Representative images of SEM. In the ClO_2 group (left column), the matrix of biofilm disappeared, indicating its integrity was destroyed. Whereas in the H_2O_2 group (right column), the biofilm structure was partially damaged. The damaged matrix structure along with the undamaged matrix structure were observed attaching to the surface of waterline. **C**. Quantitative results of VFS. The ratio of average live/dead ration of the ClO_2 group was significantly lower than that of H_2O_2 group. Data were presented as mean ± standard error, * means $p < 0.05$. CLSM: confocal laser scanning microscopy, SEM: scanning electron microscope, VFS: vital fluorescence staining.

killed (red patches representing dead bacteria, and/or the underneath linear yellow stripes representation coexistence of live and dead bacteria) in the ClO_2 group, whereas they were partially killed as uneven red patches (dead bacteria) over the bright green stripes (representing alive bacteria) in the H_2O_2 group. Active bacteria (bright green patches) almost could not be observed in the ClO_2 group, whereas could be still found in the H_2O_2 group. These findings demonstrated a better disinfecting efficiency of ClO_2 than H_2O_2 (Figure 1A). Meanwhile, the results of the SEM showed that the matrix structure of biofilms was disrupted from surface to deep layers, thereby the matrix could not be found till the bottom of the biofilm (close to the basal layer), and the bacterial body were exposed in the ClO_2 group. Whereas in the H_2O_2 group, the damage of the matrix structure was slighter, only partial surface layer and matrix were damaged (Figure 1B). Findings of CLSM were in agreements with those of SEM, indicating ClO_2, in comparison to H_2O_2, can markedly damage the surface (including the matrix) structures and infiltrate into the biofilm, thereby achieves better disinfecting effects (referred to as "penetrating effects"). Quantitative data of VFS also verified the better efficiency of ClO_2. The average live/dead bacteria ratio in ClO_2 group were significantly lower than that of H_2O_2 group (38.41% *vs.* 66.36%, U = 19/00, $p = 0.032$) (Figure 1C).

3.3. What is special issue of the ClO_2 solution used in this study?

Disinfection of DUWLs plays a key role in control and prevention of nosocomial infection in a dental clinic. It has been documented that contaminated DUWLs are risky for the patients' health (*19*), even life-threatening in some extreme cases (*20,21*). Removal and control biofilm and planktonic microbes developed in DUWLs are undoubtedly the most important tasks in terms of prevention of DUWLs contamination-related nosocomial infections (*22*). In this regard, many disinfectants were evaluated. But only several disinfectants were actually applied in the clinical setting. H_2O_2, as a high-level disinfectant, acts as the most conventional disinfectant using in the DUWLs scenario (*23*), that is recommended by the manufacturer's manual of many DUWLs makers. However, H_2O_2 is far from a faultless disinfectant in the context of a dental practice. Its unstable and irritant nature limits its further application for dental practice setting. ClO_2 is another high-level disinfectant which has been considered for using in the dental practice due to its nontoxicity and nonirritant. The limitation of ClO_2 lies in difficulties of availability of a stable and storable ClO_2 solution (*14*). The aforementioned "activation" processes are quite inconvenient and inoperable in a dental scenario because the activation concentration sometimes is difficult to control. Fortunately, a novel stable ClO_2 solution (free of activation) recently became available. Hence, using ClO_2 solution in the dental practice setting, even directly using it in human body (*14*) are becoming possible. Here, first, our CFUs study found that the disinfecting efficiency of 5 ppm of stable ClO_2 solution (free of activation) was no weaker than those of conventional 0.24% of H_2O_2 on DUWLs in actual dental practice (Table 1). During the subsequent morphological studies, we found that the 5 ppm of stable ClO_2 solution exhibited stronger disinfecting effects to biofilm at the surface of the waterline. Results of CLSM indicated that almost all patches representing live bacteria (bright green) were disappeared. Only patches representing dead bacteria (red) and coexistence of live/dead bacteria (yellow) were residual. By contrast, patches of live bacteria remained visible after disinfection with 0.24% of H_2O_2 (Figure 1A). Importantly, our CLSM data implied that ClO_2 may infiltrate into the biofilm (penetrating effects) and exhibit a better disinfection. The SEM data were in line with the CLSM data, namely 5 ppm of ClO_2 solution could completely destroy the integrity of biofilm, whereas 0.24% of H_2O_2 could only achieve a partial destroy (Figure 1B). Our data suggested that 5 ppm of ClO_2 solution displayed a stronger effect than 0.24% H_2O_2 in terms of removal/control of biofilm. The quantitative results of VFS also confirmed this finding (Figure 1C). Accordingly, the disinfecting efficiency of this stable ClO_2 solution (free of activation) was verified.

Another important issue is regarding the safety. In terms of the application scenarios of ClO_2, 5 ppm is indeed a very low dose, which is commonly used for disinfection of the fresh fruits and vegetables (*24*). As early in 1984, a human study by Lubbers *et al*. documented that no toxic reactions were found after oral intake of 5 ppm of ClO_2 (containing in the tap water) for 12 weeks (*25*). A later animal study found that no toxic effects were observed in the main organs in mice after oral administration of ClO_2 at 0-40 ppm for 90 days (*26*). By contrast, the doses of application of ClO_2 for the other scenarios commonly larger, for example, 300 ppm for disinfection of wounds with deep venous thrombosis or diabetic foot (*13*), 1,000 ppm for dental disinfection (*12*). These doses of ClO_2 directly used in human body are much greater than 5 ppm, however, are still safe. In this regard, 5 ppm of ClO_2 for DUWLs disinfection is undoubtedly safe.

3.4. Limitations and future prospects

Because the present study was designed in the scenario of dental practice setting, that means all the DUWLs were in practice every day, which required to be disinfected every day. Thus, we could not set up a "blank" control. This might be a limitation of this study. In addition, gradient experiments in different concentrations of ClO_2 and H_2O_2 are also indispensable to elucidate their destroying effects on biofilm, which

should be addressed in our future investigation.

Taken together, this pilot study conducted a comparison of the disinfecting effects on DUWLs between a commercialized stable ClO_2 solution (free of activation) and conventional H_2O_2. The present study verified the satisfactory efficiency of this stable ClO_2 solution in a low dose (5 ppm). The safe and effective nature of stable ClO_2 solution (free of activation) to biofilm indicates that it is suitable for disinfection and sterilization of DUWLs in actual dental practice.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Qiang Zhang, Department of Orthodontics, Shenzhen Stomatology Hospital, 70 Guiyuan North Street, Shenzhen, Guangdong, China 518001.
E-mail: 13500052738@163.com

Tetsuya Asakawa, Institute of Neurology, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Bulan Road, Shenzhen, Guangdong, China 518112.
E-mail: asakawat1971@gmail.com

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