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Efficacy of acoustic waves in preventing *Streptococcus mutans* adhesion on dental unit water line

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Abstract

Background. *The quality and health safety of water used for refrigeration and flushing of the handpieces, water-syringes and other components of dental units is of considerable importance. Water crosses these devices by a system of intersected small plastic tubes (about 2 mm of diameter), named dental unit water lines (DUWLs). DUWLs may be heavily colonized by many bacterial species in a planktonic phase, adherent or in biofilm lifestyle, resulting in a potential risk of infection, not only for all professionals who routinely use these devices, but also for occasional-patients, especially immunocompromised patients. Contamination of DUWLs can be prevented or reduced with the use of disinfectants, but the eradication of microorganisms, especially which those are adherent or living in biofilm lifestyle on the inner surfaces of DUWLs is challenging and often, the normal methods of water disinfection are not effective. Moreover, disinfectants routinely used to disinfect DUWLs may alter the bond strength of the dentine bonding agent used for restorative practice in dentistry.*

Study design. *To identify an innovative and alternative strategy, able to prevent bacterial adhesion to DUWL surfaces through a physical approach, which is more effective in overcoming the problem of DUWL contamination and the risk of infection compared to the standard methods already in use. In this pilot study we tested a member of the oral streptococci family, that is not a component of the biofilm detected on the walls of DUWL, but is frequently detected in water samples from DUWL, due to human fluid retraction during dental therapy. Namely, the pathogenic bacterial species Streptococcus mutans.*

Methods. *We employ elastic acoustic waves at high-energy in preventing S. mutans adhesion to the inner walls of an experimental water circuit reproducing a DUWLs. To stress the capability of acoustic waves to interfere with bacterial adhesion also in extreme conditions, a high S. mutans contamination load was adopted.*

Results. *We observe a significant decrease of adherent bacteria exposed to acoustic waves treatment respect to control.*

Conclusions. *This study demonstrates the effectiveness of acoustic waves in counteracting the adhesion of S. mutans to the inner walls of an experimental water circuit reproducing a DUWL, opening up new prospects for future practical applications. The interesting results, so far obtained, require an in-depth analysis of the methods regarding both the various bacterial species involved and the infective charges to be used.*

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List of abbreviations: DUWLs: Dental Unit Water Lines; CFUs: Colony Forming Units; PE-CFUs: Planktonic-Equivalent CFUs.

Introduction

Bacteria in water can live both in a planktonic state or as a microbial community organized in biofilm lifestyle. Bacterial biofilm may be present on the inner surfaces of dental unit water lines (DUWLs) due to contaminations coming from the proximal or distal portion of the circuit, probably as the result of inappropriate microbiological decontamination procedures (1) and it represents a potential source of infection that affects human health, in particular that of dental staff and patients, habitually exposed to water and water-aerosol emitted by dental unit handpieces (2-5).

Different approaches have been adopted to avoid DUWLs contamination and consequent risk for patient and health-professionals, and one of these does not necessarily exclude the others. Nevertheless, the microbial adhesion and biofilm on DUWL surfaces remain very difficult to eradicate (6, 7). For example, in the case of treatment with the disinfectant chlorhexidine gluconate, the disinfectant disrupted individual cells of *Porphyromonas gingivalis* but it was not able to completely remove the bacterial biofilm (8). After treatment with several disinfectants, residual adherent bacteria could be still present, thus favoring the formation of new biofilms and the persistence of risk for health (9). An additional problem, related to chemicals used to disinfect DUWLs, is that they may alter the bond strength of the dentine bonding agent used in restorative practice in dentistry (10).

Among the different methods employed to counteract microbial contaminations (5), acoustic waves at low-energy and high-frequency have been proposed for the eradication/prevention of microbial biofilm formation on catheters (11, 12).

The aim of this "small scale preliminary study" was to investigate the potential effect of high-energy low-frequency acoustic waves to counteract *Streptococcus mutans* adhesion to the inner walls of DUWLs. Indeed, among the many bacterial species, also oral streptococci are frequently detected in water from DUWLs; among these, one with excellent ability to adhere and form biofilm and a well-known causative agent of dental caries, also involved in secondary caries, is the *Streptococcus mutans* (13-15). To this purpose, we simulated the DUWL water circuit reproducing a similar one, where the *S. mutans* adhesion was tested on inner surfaces of Teflon tubes using a flow system model, both in the presence or absence of appropriately tuned high-power acoustic waves. The acoustic wave frequency "is an important factor which influences size and formation of cavitation bubbles", high-power low-frequency acoustic wave generate large cavitation bubbles producing high energy (16) when they collapse. Here, high-power acoustic waves with low-frequency (20 kHz) able to cause cavitation were used.

Materials and methods

Strain

Streptococcus mutans ATCC 25175^T strain was maintained in trypticase soy broth (Difco Laboratories, MD) with glycerol (25%) at −80°C and checked for purity on Columbia CNA agar (Difco Laboratories, MD, USA) with 5% red sheep cells before use.

Experimental model

The experimental flow model is shown in Figure 1. The flow model consisted in a bacterial culture flask, a peristaltic pump (ISMATEC MPC, Germany), an acoustic signal transducer and a waste medium-collecting flask. Teflon tubes of 2 mm diameter (inner lumen 1.8 mm) were used. Tubes were immersed in a thermostatic water-bath (25°C) equipped with an acoustic signal transducer generating low-frequency (20 KHz) and high-energy (driving pressure of 15 MPa) acoustic waves. As control, tubes were immersed in the same bath without acoustic waves. To evaluate any influence of the curvature, the portion of the tubes immersed in the water-bath generating acoustic waves were both linear or curved, where the curvature of the circuit "*K*" (see the insert at the bottom of Figure 1) is defined as the reciprocal of the radius (expressed in meters) of the osculating-circle (*K*= 6.25). To obtain a high level of bacterial colonization, tubes were flowed with a constant and continuous flow of *S. mutans* (10^7 CFU/ml) in sterile saline (0.9% NaCl in water) using the peristaltic pump (20 rpm/min; 10 ml/min). After 3 hours of flow at room temperature, tubes were washed with sterile saline for 15 minutes to eliminate the non-firmly adherent bacteria. The external surfaces of the tubes were sterilized using 70% ethanol; subsequently, ethanol was removed by washing the external surface with sterile saline solution. Immediately after, the tubes were aseptically cut into pieces of 0.5 cm length and the CFUs of the adherent bacteria in the lumen of the circuit were evaluated. In particular, the pieces were differently marked as proximal, central and distal ones, depending on the distance from the peristaltic pump (Fig. 1).

Detection of adherent *S. mutans*

Adherent bacteria on the inner surface of the Teflon tubes were counted using the BioTimer-Phenol red (BT-PR) assay, whose color changes due to microbial metabolism

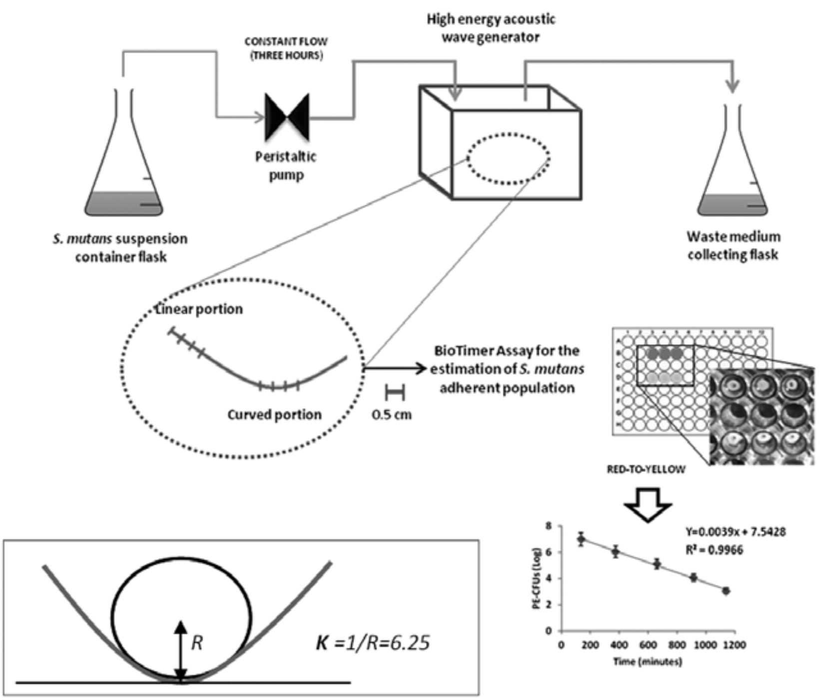

Fig. 1 - Experimental flow model. Erlenmeyer flask with *Streptococcus mutans* suspension: after acoustic waves treatment, the pipe-line ends up into discard. The time for color change (red to yellow) of BT-PR is inversely correlated to the bacterial abundance by a correlation line. The curvature of the circuit, "*K*", is defined as the reciprocal of the osculating-circle radius.

(17-20). In particular, BT-PR reagent changed red-to-yellow due to reagent acidification when inoculated with fermenting bacteria as *S. mutans*. BT-PR reagent was prepared as previously described (19). The time required for color change of BT-PR reagent is correlated to initial bacterial concentration by a correlation line. To draw the correlation line, serial two-fold dilutions of planktonic overnight broth cultures of *S. mutans* in 1 ml of BT-PR reagent was performed in 24-well plates (BD, Italy), and simultaneously counted using the CFU method. The time (in minutes) required for color changing of the inoculated BT-PR reagent was recorded and plotted versus the corresponding CFU values. The equation and the linear correlation coefficient describing the correlation line were calculated on the whole data set and were $y = -0.0039x+7.5428$ and $R^2 = 0.9966$. As the correlation line correlates the time for color change of BT-RP reagent with the number of planktonic CFUs, the number of adherent bacteria was expressed as planktonic-equivalent CFUs (PE-CFUs) (18). To evaluate the adherent bacteria, the 0.5 cm-pieces of tubes were immersed in 200 μl of BT-PR reagent in 96-well plates. The time required for color switch was checked using the TECAN SUNRISE™ (Switzerland) automated absorbance reader and used to determine the PE-CFUs/ml as above described.

Statistics

Results were derived from at least five independent experiments and reported as mean values ± SDs. Significance was calculated using Student's t-test.

Results

We evaluated the effect of acoustic waves on the adhesion of *S. mutans* using a flow model simulating dental unit water line condition. The results are shown in Table 1. Firstly, the reported data indicated that different amounts of adherent bacteria can be detected depending on the curvature of the tubes. In fact, significant lower counts in curved respect to linear portions of the tubes were noticed ($P < 0.05$). Even if further experiments are needed, this observation suggests that hydrodynamics of liquid flow in curved respect linear tubes may influence *S. mutans* adhesion similarly to what observed for biofilm development under different shear stress conditions (21). This observation may be useful in evaluating the effectiveness of disinfection and, more importantly, in the DUWL engineering. On the contrary, little differences on the effect of acoustic waves respect to control among proximal, central and distal sections in linear (91, 80, and 71% of reduction, respectively) and curved (98, 98, and 94% of reduction, respectively) portions ($P > 0.05$) were recorded. When the effect of the acoustic waves was examined on the whole data set, a significant decrease of adherent bacteria subjected to acoustic waves respect to control ($P = 0.003$) was recorded, demonstrating that acoustic waves inhibited efficiently bacterial adhesion. Moreover, when data obtained in linear and curved tubes were examined separately, significant differences were recorded ($P \leq 0.05$). In particular, the bacterial reduction of 81 and 97% in linear and curved portions, respectively, was observed. Finally, the data concerning the adherent bacteria in curved respect linear portions subjected to acoustic waves (Tab. 1) demonstrated a significant reduction of *S. mutans* ($P \leq 0.05$).

Discussion

The microbial contamination of inner wall of DUWLs mainly takes place both "from suck-back of saliva from the oral cavity occurring when a negative pressure is generated on stopping the equipment", and "from the municipal water-system

Table 1 - Adhesion of *Streptococcus mutans* onto the inner walls of Teflon tubes

	PE-CFUs (log)[a]			
	Linear portions		Curved portions	
Circuit section[b] /Treatment[c]	None	Acoustic Waves	None	Acoustic Waves
Proximal	6.47±0.79	5.41±0.56	4.21±1.92	2.46±1.50
Central	6.34±0.688	5.64±0.65	4.83±1.58	3.16±2.12
Distal	6.08±0.64	5.55±0.58	4.79±1.71	3.55±1.70
P values:				
Curved vs linear portions, no treatment	0.015			
Treatment vs no treatment (whole data set)	0.003			
Treatment vs no treatment, linear portions	0.009			
Treatment vs no treatment, curved portions	0.015			
Curved vs linear portions, treatment	0.017			

Legend: [a] the number of adherent *S. mutans* was determined using the BioTimer Assay and expressed as planktonic-equivalent CFUs (see M&M Section for details); [b]: position of the portion of the tubes respect to the flow direction; [c]: tubes were treated with acoustic waves (20KHz) for 3h.

or independent water reservoirs into the DUWL" (22). Microbial colonization leads to biofilm development that is favored by the laminar flow and the frequent stagnation periods. Dissemination of microbial aerosol generated by dental procedures is a concern for the risk of cross-infection. As consequence, the Center for Disease Control and Prevention (CDC) and the American Dental Association (ADA), 2012 guidelines (23), recommend a microbial contamination of DUWL water <500 CFU/ml (24, 25). Despite the efforts in reducing microbial contamination, this goal has not yet been reached (see for example: (6, 7, 17, 26-28).

The first step of biofilm formation on a surface is always represented by bacterial adhesion. Consequently, in general terms, the possibility to prevent bacterial adhesion could be considered a mechanism of biofilm prevention, even if, actually in our working conditions, we can't demonstrate that *S. mutans* becomes a member of the DUWLs biofilm or develops in independent biofilm lifestyle. Although the expected load of *S. mutans* in a DUWL is very low, we have stressed the efficacy test by increasing the infectious charge by many orders of magnitude and, even in these extreme conditions of contamination, the effectiveness of the acoustic waves remains significant, nevertheless to better define this concept and to strengthen its statistical value, it would be advisable to repeat these tests by serial dilutions up to below the generally detected microbial load. To avoid confounding factors and guarantee the repeatability of the experiment a sterile saline solution was used.

Our results show the effectiveness of high-energy acoustic waves in reducing *S. mutans* colonization and furthermore suggest that colonization is also influenced by the hydrodynamics of the flow. Although the decreases in the values for "PE-CFUs" after acoustic wave treatment are significant (Table 1), the values could be higher, in fact our observations on the concrete possibility of a useful synergistic effect between acoustic waves and chlorination at low concentrations (data not shown) lead us to carry on this work.

Finally, preliminary experiments on the effect of shear waves pulsed or in continuous, on pre-formed *S. mutans* biofilm indicated that low-energy (4 $\mu W/cm^2$; 2.25 MHz) acoustic waves did not decrease, but slightly increase *S. mutans* biofilm (personal data). This observation, together with the data showing the efffectiveness of high energy-acoustic waves, strongly suggests the importance in choosing the appropriate wavelength to obtain the desired effect.

This study has the following limits. First, *S. mutans* is not a colonizer of DUWL biofilm, although it is frequently detected in water samples from DUWL. The microorganisms colonizing water supplies are generally nonpathogenic environmental bacteria. However, human pathogens, such as *Pseudomonas aeruginosa*, *Legionella pneumophila* and non-tuberculosis *Mycobacterium* species are occasionally detected in DUWL biofilm. Second, the bacterial load used for the present test are largely higher than those usually detected in DUWL that can be as high as 10^5 CFU/ml, less than one hundredth the load used in the present study. Other studies are needed to test the effectiveness of this new method in real-life conditions, namely, using water biofilm bacteria at lower loads.

Conclusions

Bacterial biofilms represent a serious problem for medical devices, including dental unit water lines. With the purpose of reducing the risk of exposure to pathogenic microorganisms from dental units, municipal-water-system or independent-water-reservoir DUWLs should be subjected to microbial monitoring routinely, and exposed to decontamination protocols (6, 24). Although several options to improve DUWLs water quality have been proposed, including chemical treatments, anti-retraction device, independent water reservoirs and filters, up to now the problem of DUWLs bacterial contamination has not yet been completely solved.

The acoustic wave frequency is an important issue, which influences size and formation of cavitation bubbles. Low frequency acoustic waves (20 kHz) “transform low-energy-density elastic waves into high-energy-density collapsing bubbles” (16). As already described, elastic energy is able to dissipate bacterial clusters or flocks (12, 29). Among the many bacterial species potentially colonizing dental unit water lines, we decided to start this pilot study using the pathogenic bacterial species *S. mutans*, for its undoubted interest in the dental field and for its marked ability to adhere to and persist on inert surfaces. In this pilot study, we highlighted that acoustic wave energy prevents *S. mutans* adhesion that, although this does not mean that biofilm will be produced, it is anyway recognized as the first-step in biofilm formation. Considering a possible synergistic effect with antimicrobials (30) or other methods of disinfections, our results indicate that the use of acoustic waves, to prevent bacterial biofilms, could represent a useful and complementary-method to improve DUWL water quality. But this does not rule out that more studies, able to correlate the effectiveness of specifically tuned acoustic waves versus other bacterial species, such as *Pseudomonas aeruginosa* and *Legionella pneumophila*, are still necessary. A possible development of this research could be focused on the ability of acoustic waves, not only in preventing the adhesion of *S. mutans* to the inner lumen of dental water lines, but also to detach mature biofilms produced by the most common bacterial species normally found in DUWLs.

Practical developments: following the example of what has already been done to prevent catheter-associated urinary tract infections using acoustic waves energy (30, 31), if our preliminary data are confirmed and reproducible also on other bacterial species, it is possible to hypothesize a practical application in the field of Public Health.

Riassunto

Efficacia delle onde acustiche nel prevenire l'adesione di *Streptococcus mutans* nei circuiti idraulici dei riuniti odontoiatrici

Background. Nei riuniti odontoiatrici, la qualità dell'acqua utilizzata per la refrigerazione e il risciacquo di manipoli, siringhe e altri componenti è un aspetto di notevole importanza sanitaria. L'acqua attraversa questi dispositivi mediante un circuito interconnesso di tubi di piccole dimensioni (circa 2 mm di diametro), denominato "dental unit water line" (DUWL). I DUWL possono essere fortemente colonizzati da varie specie batteriche sia in fase planctonica, che adesi o organizzati in biofilm, rappresentando una potenziale causa di infezione, non solo per i professionisti che usano abitualmente questi dispositivi, ma anche per pazienti occasionali, in particolare per i pazienti immunocompromessi. La contaminazione dei DUWL può essere prevenuta o ridotta con l'uso dei disinfettanti, ma l'eradicazione dei microrganismi adesi alle superfici interne dei DUWL o organizzati in forma di biofilm, è una sfida assai più complessa e spesso i normali metodi di disinfezione non sono pienamente efficaci. Inoltre, in ambito odontoiatrico, i disinfettanti utilizzati abitualmente per disinfettare i DUWL possono alterare la capacità adesiva del materiale utilizzato nella pratica restaurativa.

Obiettivi. Individuare una strategia innovativa, in grado di contrastare l'adesione batterica alle superfici dei DUWL mediante un approccio di tipo fisico, che sia più efficace nel superare il problema della contaminazione dei DUWL e ridurre il rischio di infezione rispetto ai normali metodi già in uso. A tal fine, fra le molte specie batteriche potenzialmente riscontrabili nei circuiti idrici odontoiatrici, si è deciso di avviare questo studio pilota utilizzando la specie batterica patogena *S. mutans*, per il suo indubbio interesse in ambito odontoiatrico e per la sua spiccata capacità di aderire e persistere su superfici inerti.

Metodi. **U**tilizzo di onde acustiche elastiche ad alta energia nel contrastare l'adesione di *Streptococcus mutans* alle pareti interne di un circuito idrico sperimentale riproducente un DUWL. Per evidenziare l'efficacia delle onde acustiche anche in condizioni estreme, è stata utilizzata un'elevata carica contaminante di *S. mutans*.

Risultati. Si osserva una significativa riduzione dei batteri adesi soggetti a trattamento con onde acustiche rispetto al controllo (P = 0,003).

Conclusioni. Questo studio dimostra l'efficacia delle onde acustiche nel contrastare l'adesione di *S. mutans* alle pareti interne di un circuito idrico sperimentale riproducente un DUWL e apre nuove prospettive per future applicazioni pratiche. Gli interessanti risultati, fin qui ottenuti, necessitano di un approfondimento delle metodiche sia riguardo le varie specie batteriche coinvolte che le cariche infettanti da utilizzare.

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