RESEARCH REPORTS
Biological

K.F. Novak[1], M. Govindaswami[1], J.L. Ebersole[1], W. Schaden[2], N. House[3], and M.J. Novak[1]*

[1]Center for Oral Health Research, College of Dentistry, University of Kentucky, 414, Health Sciences Research Building, Lexington, KY 40536-0305, USA; [2]Trauma Center Meidling, Vienna, Austria; and [3]Tissue Regeneration Technologies, Woodstock, GA 30188, USA; *corresponding author, mjnova2@email.uky.edu

J Dent Res 87(10):928-931, 2008

Effects of Low-energy Shock Waves on Oral Bacteria

ABSTRACT

We have recently demonstrated that extracorporeal shock-wave therapy (ESWT) is effective in promoting the healing of dermal wounds and in regenerating alveolar bone lost through periodontal disease. The objective of the present study was to determine any antibacterial effect of ESWT on oral bacteria. Monoculture suspensions of 6 bacterial species were treated with 100 to 500 pulses of ESWT at energy flux densities (EFD) of 0.12 mJ/mm^2, 0.22 mJ/mm^2, and 0.3 mJ/mm^2. Following treatment, aliquots were plated for viability determination and compared with untreated controls. ESWT showed a significant microbicidal effect for *Streptococcus mutans* and an unencapsulated strain of *Porphyromonas gingivalis* following as few as 100 pulses at 0.3 mJ/mm^2 ($p \leq 0.001$). In addition, a significant disruption of bacterial aggregates was observed at lower EFDs. No significant reduction in viability was observed for all other bacteria at EFDs and pulses tested ($p > 0.05$). These findings suggest that low-energy ESWT may be bactericidal for selected oral bacteria.

KEY WORDS: shock waves, therapy, oral bacteria.

INTRODUCTION

Extracorporeal shock waves are characterized by positive pressures of up to 100 megapascals (MPa), and negative pressures of 5-10 MPa. The pressure waves travel through fluid and soft tissue, and their physical effects occur at interfaces with a change of impedance in their flow (Sturtevant, 1996). Extracorporeal shock-wave therapy (ESWT) has numerous applications in modern medicine; it is commonly used to fragment renal calculi (lithotripsy) (Fuchs and Patel, 1996), as well as to disintegrate calcium deposits in the pancreas, salivary ducts, and the gall bladder (Sauerbruch *et al.*, 1986; Delhaye *et al.*, 1992; Iro *et al.*, 1992). Focused ESWT is also used in orthopedics to facilitate bone healing and in musculo-skeletal conditions such as plantar fasciitis, symptomatic heel spur, lateral elbow pain, lateral epicondylitis, and upper limb hypertonia in people who have had strokes (Buch *et al.*, 2002; Manganotti and Amelio, 2005; Martini *et al.*, 2005; Trebinjac *et al.*, 2005; Buchbinder *et al.*, 2006; Kudo *et al.*, 2006). More recently, we have demonstrated that ESWT, when used at low energy levels, can promote healing of dermal lesions in a rodent skin flap model (Dr. H. Vasconez, personal communication) and promote the regeneration of alveolar bone in a rodent model of periodontitis (Sathishkumar *et al.*, 2008). However, we did not determine if any antibacterial effects of low-energy ESWT contributed to the clinical outcome in either of these models.

Several studies have demonstrated antibacterial effects of high-energy shock waves on planktonic micro-organisms (Kerfoot *et al.*, 1992; von Eiff *et al.*, 2000; Gollwitzer *et al.*, 2004; Gerdesmeyer *et al.*, 2005) or in association with urinary calculi (Reid *et al.*, 1990; Stoller and Workman, 1990; Prabakharan *et al.*, 1999). However, no studies to date have examined the effects of low-energy shock waves on bacteria, especially Gram-negative and Gram-positive bacteria of oral origin. The structure and rigidity of the cell walls of bacteria vary significantly, and a thin peptidoglycan layer of Gram-negative cells makes them more sensitive to disruption (Diels and Michiels, 2006). Therefore, the goal of this study was to examine the effects of low-energy ESWT on a selection of Gram-positive and Gram-negative bacteria that are frequently associated with dental plaque.

MATERIALS & METHODS

Bacterial Cultures

The bacteria used in this study were a selection of Gram-positive and Gram-negative strains that are important components of complex biofilms. Gram-negative bacteria included: *Porphyromonas gingivalis* 381 (unencapsulated, fimbriated strain), *Porphyromonas gingivalis* W83 (heavily encapsulated and essentially afimbriate strain), and *Fusobacterium nucleatum* ATCC 49256. Gram-positive strains included *Actinomyces naeslundii* ATCC 49340, *Streptococcus mutans* ATCC 25175, and *Staphylococcus aureus* ATCC

Received February 18, 2008; Last revision June 5, 2008; Accepted June 6, 2008

12600, which was included in this study as a positive control, since previous studies had shown that it was susceptible to high-energy ESWT (Von Eiff *et al.*, 2000; Gerdesmeyer *et al.*, 2005). *P. gingivalis* was grown in Difco™ Anaerobe Broth MIC,**(AQ)** and *F. nucleatum* was grown in Bacto™ Tryptic Soy Broth (TSB) at 37°C (10% H_2, 85% N_2, and 5% CO_2), and *A. naeslundii*, *S. mutans*, and *S. aureus* were grown in TSB at 37°C (5% CO_2, 5% CO_2, and air, respectively). Colonies from blood agar plates were inoculated into 5 mL of appropriate broth and grown to early-stationary phase. Cultures were then transferred to fresh media and grown to mid-logarithmic phase. We used a 0.1-mL aliquot to quantify the concentration of bacteria using a Petroff-Hausser cell counter. The cultures were then serially diluted in phosphate-buffered saline (PBS). Three milliliters of a monoculture suspension of each bacterium (1-3 x 10^5 bacteria/mL) were individually placed in pouches made from the fingertips of sterile, non-powdered surgical gloves, to simulate the interaction between bacteria and soft-tissue surfaces. Air was removed, and the opening of each pouch was tightly secured just above the level of the bacterial suspension.

Shock-wave Application

Low-energy shock waves, with energy flux densities (EFD's) of ≤ 3 mJ/mm^2, were generated electro-hydraulically with a Dermagold® unit (MTS, Konstanz, Germany). The pressure waveform for the Dermagold® unit is illustrated in Fig. 1. The approximate pressure profile values for low-energy ESWT are: peak positive pressure, 21.1 MPa; and peak negative pressure, -4.6 MPa at an EFD of 0.1 mJ/mm^2. Before shock-wave treatment, a thin film of ultrasound gel was applied to the outside of each pouch to allow for energy conduction. The bacterial suspension in each pouch was treated with 100, 200, 300, 400, and 500 pulses of ESWT. Individual pouches were treated with EFDs of 0.12 mJ/mm^2, 0.22 mJ/mm^2, and 0.3 mJ/mm^2, with a rate of 3 pulses/sec. For each EFD, and following every 100 pulses, a 0.1-mL aliquot was aseptically withdrawn and placed in a sterile Eppendorf tube, and 0.9 mL of medium was added to achieve 1-3 x 10^4 bacteria/mL. The opening to the pouch was re-secured, and the next 100 pulses were delivered. This process was continued until 500 pulses were delivered *per* sample/energy level. A 20-μL quantity of the bacterial dilution from each treatment was cultured on blood agar plates, and the viable bacteria were determined as colony-forming units (CFUs). For each bacterium, at every timepoint and energy level, untreated control pouches containing bacterial suspensions were sampled, diluted appropriately, and plated on blood agar plates. All treatment and control experiments were performed in triplicate.

Statistical Analysis

Descriptive statistics of the viable bacteria were expressed as a mean ± standard error of the mean (SEM). Comparisons of the colony-forming units (CFU) in control *vs*. treatment conditions were made by a Kruskal-Wallis one-way analysis of variance (ANOVA), followed by the Mann-Whitney Rank Sum test to determine specific differences between individual treatment conditions and the control cultures. A $p < 0.05$ was accepted as a statistically significant effect of the treatment.

RESULTS

We undertook this study to determine if low-energy shock waves could effectively decrease the viable counts of monoculture bacterial suspensions *in vitro*. We specifically targeted oral bacteria that constitute components of the plaque biofilm. Development of the plaque biofilm, as with other complex microbial biofilms, is a sequential process, with oral streptococci being the primary early colonizers. This initial colonization by streptococci is followed by increasing proportions of *Actinomyces, Veillonella, Fusobacterium* spp., and selected other species (middle colonizers), with subsequent colonization dominated by Gram-negative, anaerobic bacteria (late colonizers) (Rosan and Lamont, 2000; Kolenbrander *et al.*, 2002). We purposefully selected bacterial species representing these various stages of oral biofilm development for study, to determine their individual susceptibility prior to future studies of biofilms. In addition, we compared an encapsulated *P. gingivalis* strain W83 with the non-encapsulated *P. gingivalis* strain 381, to determine if there was a protective effect afforded by the capsule (Aduse-Opoku *et al.*, 2006). *S. aureus* was included as a positive control and to determine if the effect of low-energy ESWT was similar to that seen in earlier studies with higher-energy ESWT (von Eiff *et al.*, 2000).

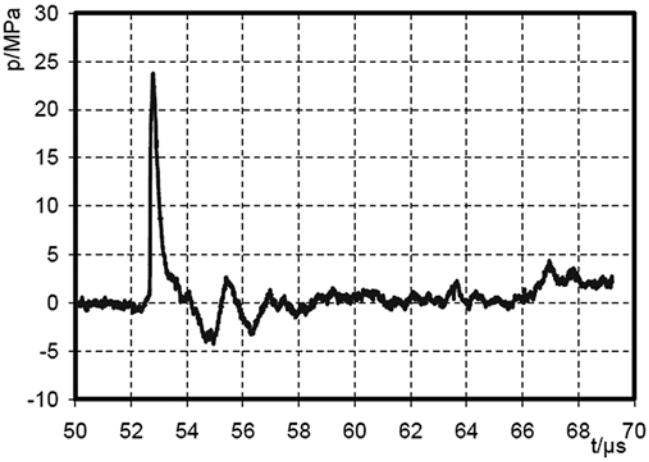

Figure 1. Pressure waveform for the Dermagold® unit at low energy. The plot shows the maximum and minimum pressures (p) created in megapascals (MPa) over time (t) in microseconds (μs).

Effect of ESWT on Gram-positive Bacteria: *S. mutans*

Viable counts for *S. mutans* in relation to the number of shock-wave pulses applied and EFD's used are expressed as a percentage of control. Baseline CFU counts corresponding to 100% of controls were 7.02 ± 0.11 x 10^4 (mean ± SEM for EFD, 0.12 mJ/mm^2), 4.4 ± 0.40 x 10^4 (for EFD, 0.22 mJ/mm^2), and 5.9 ± 1.00 x 10^4 (for EFD, 0.3 mJ/mm^2) (Fig. 2). At EFDs of 0.12 mJ/mm^2 and 0.22 mJ/mm^2, there were no significant differences between viable counts of control and respective ESWT-treated groups ($p > 0.05$). However, at an EFD of 0.3 mJ/mm^2, and with 100 to 500 pulses, there were statistically significant reductions ($p < 0.006$) in viable counts *vs*. controls and *vs*. comparable wave pulses for EFDs of 0.12 ($p = 0.025$) and 0.22 mJ/mm^2 ($p = 0.017$) (Fig. 2).

No significant changes in bacterial viability were observed at any EFD and pulse combination for any of the other Gram-positive strains tested, including *A. naeslundii* and *S. aureus*.

Effect of ESWT on Gram-negative Bacteria: *P. gingivalis* 381

The baseline CFU counts corresponding to 100% of controls were 4.01 ± 0.28 x 10^4 CFU (mean ± SEM for EFD, 0.12

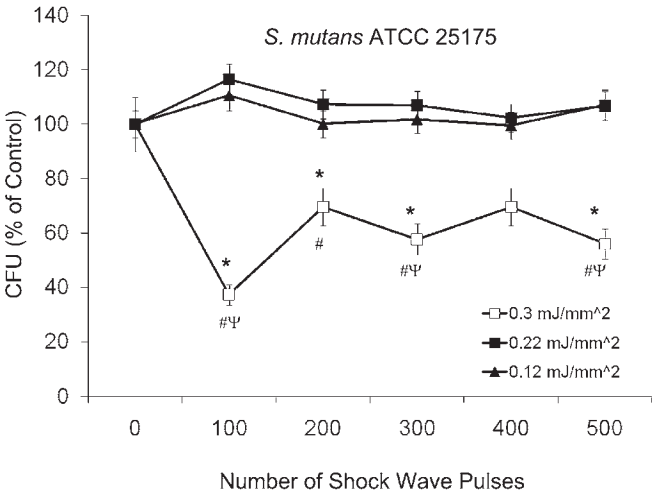

Figure 2. Effect of low-energy ESWT on *S. mutans* ATCC 25175. Colony-forming units (CFU) are expressed as a percentage of controls in relation to number of shock-wave pulses and energy flux density (EFD). Data are expressed as the mean ± standard deviations of triplicate experiments. *Indicates a significant ($p < 0.006$) decrease in CFU *vs.* controls; #indicates a significant ($p = 0.017$) decrease in CFU *vs.* corresponding pulse point at EFD 0.22 mJ/mm^2; and ψ indicates a significant ($p = 0.025$) decrease in CFU *vs.* corresponding pulse point at EFD 0.12 mJ/mm^2.

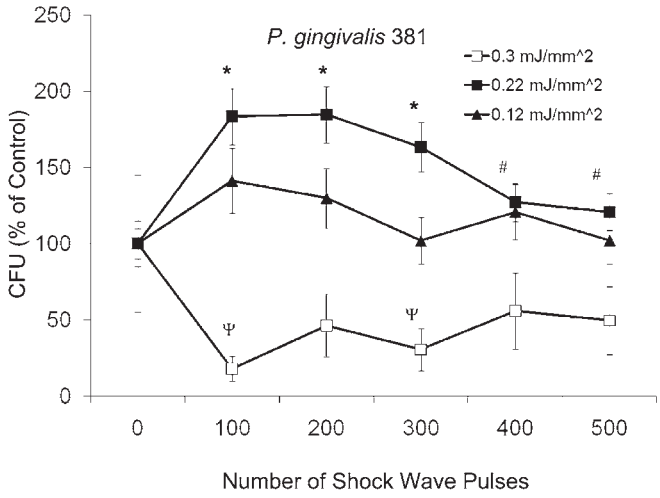

Figure 3. Effect of low-energy ESWT on *P. gingivalis* 381. CFU are expressed as a percentage of the control in relation to number of shock-wave pulses and EFD. Data are expressed as the mean ± standard deviations of triplicate experiments. *Indicates a significant increase in CFU ($p < 0.006$) *vs.* controls at EFD 0.33 mJ/mm^2; #indicates a significant decrease in CFU ($p < 0.006$) *vs.* 100 and 200 pulses at EFD 0.22 mJ/mm^2; ψ indicates a significant decrease in CFU at EFD 0.3 mJ/mm^2 ($p < 0.006$) *vs.* corresponding pulse point at EFD 0.22 mJ/mm^2.

mJ/mm^2), 3.79 ± 0.40 x 10^4 CFU (for EFD, 0.22 mJ/mm^2), and 1.58 ± 0.37 x 10^4 CFU (for EFD, 0.3 mJ/mm^2) (Fig. 3). At EFD 0.12 mJ/mm^2, no significant changes in viable counts were seen following any number of pulses. In contrast, at EFD 0.22 mJ/mm^2, there was a statistically significant increase in CFUs over control for pulses of 100 to 300 ($p < 0.006$), due to disaggregation of cells that commonly clump with this strain, due to their lack of capsule and, therefore, increased hydrophobicity in liquid suspension. As the number of shock-wave pulses increased at EFD 0.22mJ/mm^2, there was a significant reduction at 400 and 500 pulses ($p < 0.006$), compared with 100 and 200 pulses, suggesting that there was a decrease in the viability of some cells at higher pulse rates. As the EFD increased to 0.3 mJ/mm^2, there continued to be significant reductions in viability when compared with CFUs at an EFD of 0.22 mJ/mm^2 ($p \leq 0.017$).

No significant changes in bacterial viability were observed at any EFD and pulse combination for any other Gram-negative strain tested, including the encapsulated strain, *P. gingivalis* W83, and *F. nucleatum*.

DISCUSSION

Previous studies have shown that the delivery of energy, through ultrasound, to bacteria in suspension results in a two-phase response (Joyce *et al.*, 2003). At low power, there is an initial rise in cell numbers as a result of disaggregation of bacterial agglomerates, whereas at high power, there may be an initial rise in numbers from disaggregation, followed by falls in viable cell numbers as the disaggregation finishes and bacterial killing takes over. Ultrasound can inactivate bacteria and disaggregate bacterial clusters through several mechanisms arising from acoustic cavitation (Joyce *et al.*, 2003). Energy released from cavitation bubbles may affect bacterial viability through direct effects on cell membranes, shear forces, and possible chemical attack through the formation of oxygen radicals. The effects of shock waves occur primarily at sites of impedance change in their flow. High-energy ESWT, at EFDs as high as 0.96 mJ/mm^2, has been effectively used for the *in vitro* killing of bacteria (von Eiff *et al.*, 2000; Gollwitzer *et al.*, 2004; Gerdesmeyer *et al.*, 2005). It is clear that bacterial killing is a function of energy level and impulse frequency, and that these variables may differ for different bacteria (Joyce *et al.*, 2003).

In the current study, the impact of low-energy ESWT, at EFDs of ≤ 0.3 mJ/mm^2, on Gram-positive and Gram-negative bacteria was assessed. As previously reported (Joyce *et al.*, 2003), at low EFDs and pulse numbers, disruption of bacterial aggregates and mother/daughter cells was seen, especially with *P. gingivalis* 381, even though cell suspensions were aggressively mixed by vortexing prior to being plated. In contrast, at the highest EFD, significant decreases in viable counts were noted with the Gram-positive bacterium, *S. mutans*, and the unencapsulated Gram-negative strain, *P. gingivalis* 381. However, the killing effect observed for these two oral strains at an EFD of 0.3 mJ/mm^2 was not observed for *S. aureus*, which has been readily killed in previous studies with a high EFD of 0.9 mJ/mm^2 combined with a high pulse frequency. *S. mutans* was reduced in numbers by approximately 50% at an EFD of 0.3 mJ/mm^2, from a baseline CFU count of 5.9 ± 1.00 x 10^4. *P. gingivalis* was reduced in viability by > 50% from a baseline CFU count of 1.58 ± 0.37 x 10^4 at an EFD of 0.3 mJ/mm^2. The killing of both bacteria compares favorably with that seen with high-energy ESWT.

In addition to the use of low-energy ESWT in this study *vs.* the high-energy ESWT in previous studies, other differences in study design should be noted. In this study, both the sample volume and the material holding the sample differed from those

used in evaluation of the impact of high-energy ESWT on bacterial viability. This change in study design was intentional, since we attempted to simulate a soft-tissue/fluid interface. Also, the volume used allowed us to withdraw a 0.1-mL aliquot following every 100 pulses without significantly altering the total volume remaining for treatment. In addition, the highest EFD used in this study was 0.3 mJ/mm^2 *vs*. the 0.9 mJ/mm^2 used in previous studies of ESWT. This difference is based upon the capabilities of the Dermagold® shock-wave generator, which is specifically designed to generate low-energy ESWT to stimulate the resolution of inflammation, tissue repair, and regeneration. We also limited the number of pulses to 500, since the study was designed to approximate treatment that may be provided in a clinical setting.

While it appears from this initial study that low-energy ESWT may not predictably lead to the efficient killing of the majority of Gram-negative or Gram-positive micro-organisms in the sample volumes tested in this study, it has the potential to disrupt bacterial aggregates found in dental/medical pathogenic biofilms. This exciting potential for the non-invasive mechanical disruption of biofilms could facilitate the use of other adjunctive antibacterial treatments, such as antimicrobials and/or antibiotics, or even provide enhanced access to the biofilm components by endogenous antimicrobial peptides and/or specific antibodies (Otto, 2006). To assess this potential, additional *in vitro* experiments in complex microbial biofilms and *in vivo* studies in animal models will need to be conducted.

In summary, these studies demonstrated that low-energy ESWT is capable of disaggregating the Gram-positive and Gram-negative bacteria that are important in biofilm formation, and of selectively killing two of the primary pathogens associated with oral and systemic infections. The limited killing observed in this study may have been due to the low levels of energy and the relatively high volume of target planktonic bacteria. Future studies will focus on the application of this low-energy, non-invasive technology, to complex biofilm formation on solid surfaces.

ACKNOWLEDGMENTS

This study was funded by a grant from Tissue Regeneration Technologies (Woodstock, GA, USA) to Dr. K.F. Novak. Drs Schaden and House are employees of Tissue Regeneration Technologies. No other consultant fees or financial arrangements exist between the authors and the sponsors.

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