Extracorporeal Shock Wave Therapy Induces Alveolar Bone Regeneration

INTRODUCTION
Periodontitis is an immuno-inflammatory disease that leads to destruction of periodontal ligament and adjacent supporting alveolar bone, and is induced by pathogenic subgingival microbial biofilms containing several periodontal pathogens, including P. gingivalis. P. gingivalis is a major component of these biofilms in severe forms of human periodontal disease, and possesses multiple metabolic properties and likely virulence factors consistent with its pathogenic role in the disease (Kuramitsu, 2003; Holt and Ebersole, 2005). P. gingivalis, as an asaccharolytic micro-organism, elaborates several different proteases that have been suggested to contribute to bacterial virulence by in vitro studies (Gibson and Genco, 2001; Yagishita et al., 2001; Chen et al., 2002). Numerous studies have used both mouse and rat models to establish short-duration infections with periodontal micro-organisms that support their virulence potential (Baker et al., 2000; Taubman et al., 2005; Kesavalu et al., 2006, 2007a). Generally, these studies have confirmed the potential of these pathogens to elicit inflammation and tissue destruction in the host (Madden and Caton, 1994).

Extracorporeal shock waves are high-energy acoustic waves generated under water with high-voltage explosion and vaporization. The application of shock wave therapy in humans has been primarily to disintegrate kidney stones, induce neovascularization to promote tissue regeneration, heal non-union long bone fractures, dissolve calcified tendonitis of the shoulder, and treat lateral epicondylitis of the elbow, proximal plantar fasciitis, avascular necrosis of the femoral head, and patellar tendonitis (Haupt, 1997; Wang, 2003). Several studies confirmed a positive effect of shock wave therapy resulting in therapeutic outcomes for musculoskeletal disorders are not fully understood. Recently, it has been shown that shock waves stimulate the early expression of angiogenesis-related growth factors, including endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and proliferating cell nuclear antigen (PCNA), contributing to induced vascularization and improving blood supply, with increased cell proliferation and tissue regeneration and repair (Wang et al., 2002; Wang, 2003). Moreover, the induction of angiogenic biomolecules and clinical observations of vascularization kinetics indicate that shock wave therapy is dose-dependent and that symptoms improve over time (Wang et al., 2002; Wang, 2003). Several in vivo studies confirmed a positive effect of shock wave treatment on fracture healing in rats, promotion of bony union, enhanced callous formation, and induction of cortical bone formation in dogs (Haupt et al., 1992; Johannes et al., 1994; Wang et al., 2001).

Several in vitro studies have shown that shock waves had a bactericidal effect on Streptococcus aureus, Streptococcus epidermidis, Pseudomonas aeruginosa, and a MRSA 27065 strain (von Eiff et al., 2000; Gerdesmeyer et al., 2005), with decreases of over 3-logs for certain species. Gerdesmeyer et al. (2005) evaluated the bactericidal activity on S. aureus as a function of...
energy flux density (EFD) and impulse number, to determine optimal in vivo conditions.

While many studies have emphasized the beneficial effects of shock wave therapy in musculoskeletal tissue and bone regeneration, no reports to date have determined its effect on the healing of periodontal tissues and alveolar bone resorption that results from the chronic inflammation of periodontitis. The hypothesis tested in this investigation was that extracorporeal shock wave therapy could promote the healing of periodontal tissues following the induction of periodontitis by *P. gingivalis* infection in a rat model of periodontitis.

**MATERIALS & METHODS**

**Bacteria**

*P. gingivalis* strain 381 was used in this study, and cultured and maintained for the animal infections as described previously (Kesavalu et al., 2006, 2007a,b).

**Rats**

Female Sprague-Dawley rats (8-9 wks old, Harlan, Indianapolis, IN, USA) were maintained under micro-isolator conditions, fed standard powdered chow (Teklad Global 18% protein rodent diet 2918, Harlan Teklad, Madison, WI, USA), and H₂O *ad libitum*, and were kept at 25°C with alternating 12-hour periods of light and dark. All procedures were performed in accordance with the approved guidelines set forth by the Institutional Animal Care and Use Committee at the University of Kentucky. Following acclimation (1 wk), rats were randomized into 5 groups [(forward) 5′ GGT AAG TCA GCG GTG AAA CC 3′ and (reverse) 5′ ACG TCA TCC ACA CCT TCC TC 3′]. After denaturation at 94°C for 5 min, 35 PCR cycles were performed, with each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, 60 sec of polymerization at 72°C, and final extension at 72°C for 7 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide for detection of a 601-bp product.

**ESWT Administration**

Extracorporeal shock waves were generated with a DermaGold® (MTS, Konstanz, Germany). Rats were anesthetized with isoflurane inhalation anesthesia and shaved on both cheeks. A thin film of ultrasound gel was applied, and unfocused shock waves were applied equally on both cheeks in a single session. After 10 wks of infection and induction of periodontitis, rats were treated with a single application of 100, 300, or 1000 impulses of unfocused ESWT at EFD 0.1 mJ/mm². The pulse rate applied was 5 pulses per sec. Rats were then killed at 0 (baseline), 3, 6, and 12 wks post-ESWT for evaluation of the potential effects of ESWT on the healing of periodontal tissues. Skulls were removed, autoclaved, and de-fleshed, and maxillae and mandibles were hemi-sected for radiographic evaluation of alveolar bone resorption.

**Radiographic Assessment of Alveolar Bone Resorption**

Hemi-sected maxillae and mandibles were trimmed to reduce the bucco-lingual dimensions to allow the teeth to be close to the radiographic film. Each jaw was secured, by means of rope wax, to a Kodak Ultra Speed size 2 films (Kodak, Rochester, NY, USA), and a Planmeca Prostyle Intra X-ray unit (Planmeca, Roselle, IL, USA) was placed at a right angle to the film. Each jaw was radiographed with an exposure time of 0.05 sec at a setting of 70 KvP and 8 mA. Radiographs were analyzed for alveolar bone height as the primary

**Bacterial Infection**

All rats were administered kanamycin (20 mg) and ampicillin (20 mg) daily for 4 days in the drinking water, and the oral cavity was swabbed with 0.12% chlorhexidine gluconate (Periogard: Procter & Gamble, Cincinnati, OH, USA) mouthrinse, to suppress the oral microbiota (Kesavalu et al., 2006, 2007a,b). *P. gingivalis* cells at 2 x 10ⁱ⁰ per mL were mixed with equal amounts of sterile 2% carboxymethylcellulose (CMC; Sigma Chemical Co., St. Louis, MO, USA) and used to infect rats orally within 15 min of removal from the anaerobic environment.

**PCR Analysis**

Rats were orally infected with approximately 10ⁱ⁰ *P. gingivalis* for 4 consecutive days on 4 alternate wks during the study period (Fig. 1). In total, 4 post-infection microbial samples were collected from all *P. gingivalis*-infected rats, by means of sterile cotton swabs at 1, 5, 7, and 9 wks post-infection. The swabs were suspended in 50 μL of Tris-EDTA (TE) buffer. The oral microbial samples were boiled in TE buffer with an equal volume of 1 N NaOH, and DNA was dissolved in 40 μL of sterile distilled water (Kesavalu et al., 2006, 2007a,b). Polymerase chain-reaction (PCR) was carried out in 50 μL volume with 10 pM of primer, and 1 mM of each deoxynucleotide triphosphate (AQ) and 1.5 mM MgCl₂. A two-unit quantity of Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA) in the manufacturer’s buffer was used with a GeneAmp PCR System. The PCR oligonucleotide species-specific primer used 16S rDNA for *P. gingivalis*: (forward) 5′ GGT AAG TCA GCG GTG AAA CC 3′ and (reverse) 5′ ACG TCA TCC ACA CCT TCC TC 3′. After denaturation at 94°C for 5 min, 35 PCR cycles were performed, with each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, 60 sec of polymerization at 72°C, and final extension at 72°C for 7 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide for detection of a 601-bp product.

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outcome parameter of the study. We used radiographs projected at a 5x size to obtain linear measures from the cemento-enamel junction to the bone height at mesial and distal interproximal surfaces (2 sites per tooth) of each of the 2 molars and 1 premolar in each quadrant (Reed and Polson, 1984; Kesavalu et al., 2006, 2007a). So that comparability of the results could be ensured, the measures were determined by two investigators blinded as to the group designation, evaluating a given radiograph blindly, and two replicate evaluations were done. Approximately 5-10% of variability was observed between investigators doing the readings and routinely calibrated with a set of standard radiographs from the rats. The summation of bone resorption in mm was tabulated and analyzed for intra- and inter-group differences.

**Statistical Analysis**

Descriptive evaluation of the alveolar bone data is presented as mean ± SD. Statistically significant differences between the groups were determined by an ANOVA and the Holm-Sidak post hoc multiple-comparisons test (SigmaStat 3.0, SYSTAT Software Inc., Chicago, IL, USA) for normally distributed data. Data determined to be non-normal were analyzed by Kruskal-Wallis ANOVA on ranks, and multiple comparisons were adjusted by Dunn’s method (SigmaStat 3.0).

**RESULTS**

PCR evaluation of the oral microbial samples collected at 4 timepoints demonstrated that 85-100% of rats (Gr I, 20/24; Gr II, 18/18; Gr III, 17/18; Gr IV, 16/18; Gr V, 0/24) were infected with *P. gingivalis* during the experimental periodontal disease period. None of the uninfected control rats was positive for *P. gingivalis* infection during the study period. The *P. gingivalis*-infected and uninfected control rats were normal and healthy following the single application of ESWT on both their cheeks. The body weights of rats following ESWT application were unchanged in both *P. gingivalis*-infected and uninfected control rats (data not shown).

*P. gingivalis* infection resulted in significantly increased maxillary, mandibular, and total alveolar bone resorption at 10-18 wks post-infection compared with uninfected control rats (Fig. 2). This was an overall loss in bone height, with associated circumferential angular defects. All measures were taken to the most coronal level of the supporting level of alveolar bone on the mesial and distal surfaces of the study teeth. In contrast, 3 wks following ESWT treatment at 300 and 1000 impulses in infected animals (Figs. 2, 3), maxillary, mandibular, and total alveolar bone levels were significantly improved compared with those in untreated and infected controls (Fig. 3, Table). Furthermore, ESWT administration at 300 and 1000 impulses continued to demonstrate a significant improvement in alveolar bone height at 6 and 12 wks post-treatment that was most notable in the maxillary bone (Figs. 2, 3). A summary assessment of a single application of ESWT, showing a dose-response effect for increased pulse number over time for maxillary, mandibular, and total alveolar bone, is given in the Table.

**DISCUSSION**

This study demonstrated our ability to colonize rats orally with *P. gingivalis* for an extended interval, to quantify the resulting alveolar bone resorption from this oral infection, and to determine the effect of ESWT on the regeneration of alveolar bone.
Table. Statistical Evaluation of ESWT Effects on Alveolar Bone Levels Related to Numbers of Impulses and Times of Evaluation

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Weeks post-ESWT, P =</th>
<th>No. of Impulses</th>
<th>No. of Impulses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Maxillary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (untreated) Gr V</td>
<td>100 Gr II</td>
<td>0.128*</td>
<td>NS</td>
</tr>
<tr>
<td>0 (untreated) Gr V</td>
<td>300 Gr III</td>
<td>0.0021</td>
<td>0.006</td>
</tr>
<tr>
<td>0 (untreated) Gr V</td>
<td>1000 Gr IV</td>
<td>&lt;0.001</td>
<td>0.0028</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
<td>0.053</td>
<td>NS</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>&lt;0.0018</td>
<td>NS</td>
</tr>
<tr>
<td>300</td>
<td>1000</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mandibular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.095</td>
<td>NS</td>
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<td>300</td>
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</tr>
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<td>1000</td>
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</tr>
<tr>
<td>100</td>
<td>300</td>
<td>NS</td>
<td>NS</td>
</tr>
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<td>1000</td>
<td>0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>300</td>
<td>1000</td>
<td>0.0033</td>
<td>NS</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>0.0386</td>
<td>NS</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.0007</td>
<td>0.0031</td>
</tr>
<tr>
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<td>&lt;0.001</td>
<td>0.0026</td>
</tr>
<tr>
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<td>300</td>
<td>0.059</td>
<td>NS</td>
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<tr>
<td>100</td>
<td>1000</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>300</td>
<td>1000</td>
<td>0.0076</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Values indicate P-value for comparison. Bold values indicate significant difference. NS denotes not significant. 0 denotes animals were not treated (Group V) with extracorporeal shock waves. Comparisons were made between untreated group (Gr V) vs. treated groups at 100 (Gr II), 300 (Gr III), and 1000 (Gr IV) impulses in maxillary, mandibular, and total alveolar bone levels. Also comparisons were made between 100 impulses vs. 300 & 1000 impulses and 300 vs. 1000 impulses in maxillary, mandibular, and total alveolar bone levels.

have been lost during the initial 10 wks of *P. gingivalis* infection (i.e., untreated, infected rats). It remains to be determined how rapidly the effect can be visualized, but future studies could estimate the potential for “regeneration” of the resorbed bone at 1-2 wks after ESWT administration. Moreover, the experimental design demonstrated a dose-response effect, showing greater bone height (e.g., regeneration) with increasing pulse number. The effects of ESWT on maxillary, mandibular, and total bone regeneration were clearly and significantly evident at 3 wks for all ESWT groups and for all pulse numbers after the single application of ESWT. There was a general tendency for this regenerative effect on bone levels to last for at least 6 wks after the single ESWT application of 300 and 1000 impulses, although the effect was not sustained at 100 impulses for bone levels at 6 and 12 wks. It is also clear, however, that the effects on bone destruction of oral infection with *P. gingivalis* continued for the 12 wks post-ESWT, with many of the rats showing bone levels at 12 wks post-ESWT that were not significantly greater than levels in untreated rats. This suggests that *P. gingivalis* infection in the rats persisted past the 12-week timepoint and continued to promote inflammation and bone resorption that ultimately overcame the beneficial effects of the single treatment with ESWT. The kinetics of the response to ESWT suggests that additional applications would likely enhance the alveolar bone regeneration lost during periodontal infections. This would be consistent with the beneficial effects of ESWT on tissue regeneration seen in previous human studies. The ability to identify various therapeutic modalities that could regenerate periodontal tissues lost through destructive periodontitis would be an important contribution to the repertoire of therapies used to manage chronic periodontal disease. This report provides initial data suggesting that ESWT may be of use in the management of periodontal bone loss. Further studies are required to determine the effects of ESWT on mechanisms of alveolar bone regeneration, on critical biomolecules in periodontal tissues, and on outcomes when used as an adjunct to conventional periodontal treatment.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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