Periodontal disease involves multiple interactions between bacterial biofilms and inflammatory responses of the periodontal connective tissue. Several MMPs play important roles in periodontal tissue breakdown by initiating extracellular matrix degradation. Tetracyclines can have therapeutic value by improving periodontal status through inhibition of MMP activity, independent of any antimicrobial properties. For this reason, SDDs have been intensively investigated for the treatment of chronic periodontitis in human medicine. The targeted plasma concentration for an SDD is recommended to be < 1 µg/mL, which is the MIC in humans.

Objective—To identify a subantimicrobial dose of doxycycline hyclate (SDD) and for the treatment of periodontitis in dogs.

Animals—20 healthy Beagles for measurement of serum doxycycline concentration and 15 Beagles with periodontitis for evaluation of the efficacy of the SDD.

Procedures—5 dogs each received doxycycline hyclate PO at a dose of 1, 2, 3, or 5 mg/kg. Blood samples were collected before and after administration, and serum concentrations of doxycycline were measured via high-performance liquid chromatography. Mean serum doxycycline concentrations were calculated, and SDDs were identified. In a separate trial, the identified SDDs (1 or 2 mg/kg) were administered PO once a day for 1 month to dogs with periodontitis (n = 5/group) and a control group (5) was fed vehicle only during the same period. Degree of gingival attachment and bleeding on probing (present or absent) were recorded. Gingival samples were collected before and after the 1-month period from the same anatomic sites. Degree of matrix metalloproteinase inhibition in gingival samples was determined via gelatin zymography and compared among treatment groups.

Results—Mean serum doxycycline concentrations in healthy dogs that received 1 or 2 mg of doxycycline/kg were consistently significantly lower than the minimal inhibitory doxycycline concentration for treatment of periodontitis throughout the 24-hour posttreatment period. Zymographic intensities were lower in dogs given 1 and 2 mg/kg than in the control dogs, and the degree of gingival attachment and bleeding significantly improved in dogs given 2 mg/kg, compared with in the control dogs and dogs given 1 mg of doxycycline/kg.

Conclusions and Clinical Relevance—A doxycycline dosage of 2 mg/kg daily appeared to be an appropriate subantimicrobial regimen for dogs with periodontitis. Furthermore, this dosage may be suitable for long-term treatment of gelatinolytic inflammatory diseases such as periodontitis in this species. (Am J Vet Res 2013;74:130–135)
Several analytic methods have been developed for the measurement of serum concentrations of antimicrobials in various species of animals. High-performance liquid chromatography is rapid, simple, and sufficiently sensitive to determine this concentration in microsamples of serum and has been successfully used in experimental studies. The purpose of the study reported here was to identify the optimal SDD of doxycycline for treatment of periodontitis in dogs. The desired dosing regimen would not result in a serum doxycycline concentration that exceeded the MIC but would result in clinical improvement through a hypothesized inhibitory effect on MMP activity in inflamed periodontal tissues.

Materials and Methods

Animals—Twenty healthy Beagles (approx 1.5 years old) were used to identify an SDD. Fifteen 3- to 5-year-old Beagles with moderate to severe periodontitis were confirmed to be otherwise healthy through a physical examination. Dogs were excluded from either portion of the study if they had been treated systemically with any medication during the 2 weeks prior to the study. The study protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University.

SDD identification—The 20 healthy Beagles were randomly allocated to receive doxycycline hyclate at a dose of 1, 2, 3, or 5 mg/kg (n = 5 dogs/group). Doxycycline was contained in a gelatin capsule and was administered orally after 6 hours of food withholding. Blood samples (1.7 mL) were collected via a 23-gauge needle from a jugular vein before doxycycline administration and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 hours after drug administration. The blood was allowed to clot in a serum-separating tube, and serum was harvested by centrifugation at 1,300 × g for 10 minutes.

A stock solution of doxycycline for standards preparation was made by dissolving 10 mg of doxycycline in 10 mL of untreated canine serum. Five standard solutions were prepared by further dilution of the stock solution with untreated serum to produce solutions containing 0.49, 0.98, 1.95, 3.91, and 7.81 µg of doxycycline/mL. The serum and standard solutions were stored at −20°C until analyzed.

Table 1—Mean ± SD serum doxycycline concentrations (µg/mL) in healthy Beagles (n = 5/group) before and at various points after administration of 1 dose of doxycycline hyclate at 1, 2, 3, or 5 mg/kg, PO.

<table>
<thead>
<tr>
<th>Measurement point (h)</th>
<th>1 mg/kg</th>
<th>2 mg/kg</th>
<th>3 mg/kg</th>
<th>5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>0.26 ± 0.44</td>
<td>0.25 ± 0.04</td>
<td>0.30 ± 0.08</td>
<td>1.65 ± 1.77</td>
</tr>
<tr>
<td>1</td>
<td>0.31 ± 0.56</td>
<td>0.48 ± 0.72</td>
<td>1.46 ± 0.66</td>
<td>6.59 ± 5.71</td>
</tr>
<tr>
<td>1.5</td>
<td>0.33 ± 0.49</td>
<td>0.53 ± 0.79</td>
<td>2.08 ± 0.46</td>
<td>9.68 ± 8.60</td>
</tr>
<tr>
<td>2</td>
<td>0.30 ± 0.47</td>
<td>0.64 ± 0.73</td>
<td>2.71 ± 0.98</td>
<td>10.95 ± 9.30</td>
</tr>
<tr>
<td>3</td>
<td>0.32 ± 0.53</td>
<td>0.70 ± 0.64</td>
<td>3.13 ± 1.35</td>
<td>12.14 ± 10.11</td>
</tr>
<tr>
<td>4</td>
<td>0.32 ± 0.50</td>
<td>0.79 ± 0.67</td>
<td>2.95 ± 1.55</td>
<td>11.24 ± 8.24</td>
</tr>
<tr>
<td>5</td>
<td>0.28 ± 0.43</td>
<td>0.76 ± 0.57</td>
<td>2.44 ± 1.32</td>
<td>10.77 ± 8.57</td>
</tr>
<tr>
<td>6</td>
<td>0.27 ± 0.42</td>
<td>0.66 ± 0.57</td>
<td>2.33 ± 1.20</td>
<td>10.93 ± 7.45</td>
</tr>
<tr>
<td>8</td>
<td>0.24 ± 0.37</td>
<td>0.59 ± 0.61</td>
<td>1.99 ± 0.99</td>
<td>8.41 ± 5.47</td>
</tr>
<tr>
<td>12</td>
<td>0.27 ± 0.37</td>
<td>0.47 ± 0.48</td>
<td>1.94 ± 0.89</td>
<td>6.04 ± 4.98</td>
</tr>
<tr>
<td>24</td>
<td>0.08 ± 0.19</td>
<td>0.37 ± 0.56</td>
<td>0.85 ± 1.00</td>
<td>2.41 ± 1.59</td>
</tr>
</tbody>
</table>

Figure 1—Mean serum doxycycline concentrations in healthy Beagles (n = 5/group) after oral administration of a 1, 2, 3, or 5 mg/kg dose of doxycycline hyclate. The horizontal dashed line represents 1 µg/mL.
Clinical and biochemical effects of SDD on periodontitis—In each of the 15 healthy dogs, 1 to 3 premolars and molars were selected for examination on the basis of existence of CAL (≥ 1 mm/tooth) or BoP (≥ 0.33/tooth). Degree of CAL was evaluated by measurement of the distance between the cementoenamel junction and the bottom of probeable pocket.16 Bleeding on probing was scored as absent or present within 10 seconds after probing.16

The dogs were randomly allocated to 3 groups of 5 dogs (10 teeth) each: administration of doxycycline at a dosage of 1 or 2 mg/kg/d or administration of vehicle only (control group). All treatments were administrated PO once a day, 30 minutes after every morning meal. The clinical condition of each dog was ascertained daily. Periodontal status including CAL and BoP was evaluated 4 weeks after doxycycline administration began at the mesial-buccal, buccal, and distal-buccal gingival margins of each tooth. All measurements were made by an experienced clinician (SEK), who used a periodontal probe.i

For evaluation of the regional MMP inhibitory effect by the systemic administration of doxycycline, gelatin zymography was performed with full-thickness gingival tissue. Tissue sample collection was performed at 1 to 3 oral sites in each dog. Before and 4 weeks after daily doxycycline administration began, tissue samples (approx 1 × 2 mm/sample) were obtained from the buccal gingival margin. Samples were washed immediately with cold distilled water (4°C) to remove blood and debris and stored at −80°C until analyzed. Thawed gingival tissue samples were weighed, and protein was extracted from the tissues at 4°C with lysis buffer blended with protein cocktailh (10 mg [wet weight] of gingival tissue/100 µL of buffer). Protein in the gingival extracts was quantified with the Bradford method.17 Twenty-five micrograms of extracted tissue proteins was mixed with the same volume of 2X renaturing buffer for 30 minutes at room temperature (approx 20°C) with gentle agitation and equilibrated in 1X developing bufferi without heat denaturation.

Electrophoresis was performed with a 10% zymogram gel containing 0.1% gelatin at 125 V for 95 minutes. After electrophoresis, the gels were incubated in developing bufferj containing 5% gelatin at 12°C for 30 minutes at room temperature (approx 35°C) with gentle agitation and destained with 0.1% Coomassie brilliant blue R250n and destained with 10% acetic acid in 40% methanol. Pre-stained protein markersk were run on each gel to identify the molecular size of gelatinase included in the samples. The intensities of the destained bands were determined with a software programl following the gel-scanning process with a luminescent image analyzer.l

Statistical analysis—Serum doxycycline concentrations in each group are reported as mean ± SD. Statistical analyses were performed with a commercial software program.7 To evaluate differences in serum doxycycline concentration change within a treatment, data were analyzed via repeated-measures ANOVA. The correlation between the amount of orally administered doxycycline and the maximum doxycycline concentration in serum was assessed by calculation of the Pearson correlation coefficient (r).

To evaluate the clinical effect of SDD, changes in periodontal status from weeks 0 through 4 in the same group were assessed via a paired t test. One-way ANOVA was used for the intergroup comparison of the periodontal values at weeks 0 and 4 and the variance of gelatinolytic activity between weeks 0 and 4. Baseline gelatinolytic values (week 0) were also compared via 1-way ANOVA to evaluate the difference among the 3 treatment groups. The Tukey method was used as a post hoc test, and 95% confidence intervals or values of P < 0.05 were used to indicate significant differences.

Results

SDD identification—Changes in the mean serum doxycycline concentration in healthy dogs before and after doxycycline hyclate administration were summarized (Table 2). The mean ± SD HPLC retention time for doxycycline was 2.94 ± 0.19 minutes. Serum doxycycline concentrations reached a maximum 3 to 4 hours after administration in all groups and remained lower than the doxycycline MIC for treatment of periodontitis for 24 hours in dogs that received the 1 or 2 mg/kg doses (Figure 1). Repeated-measures ANOVA revealed significant differences in the mean serum doxycycline concentration within each group according to the time flow. The correlation between dose administered and detectable serum concentration was significant (r = 0.72).

Clinical and biochemical effects of SDD on periodontitis—As a result of findings in the preliminary portion of the study, the SDD chosen for further evaluation was 1 and 2 mg/kg, once a day. Values of periodontal variables, including CAL and BoP, were not significantly different among the groups at week 0 (before treatment began). After the 4-week medication period, dogs that received doxycycline at a dosage of 2 mg/kg/d had a significantly lower degree of CAL, compared with the value at week 0 and that for dogs that received a placebo or a 1 mg/kg/d dosage (Table 2). On week 4, the BoP score of dogs that received 2 mg/kg/d was significantly lower than at week 0, whereas those for other groups were higher but not significantly.

In the zymographic evaluation, gelatinolytic bands were evident in extracts from all gingival samples at the 92- and < 72-kDa areas (Figure 2). Gelatinolytic i...
The intensities at both molecular weights increased in the control group after 4 weeks of treatment, whereas in the other groups, a decrease in intensity was evident after treatment concluded. The variation was significantly different in the control group, compared with in the other groups (Table 3).

Discussion

Tetracyclines are generally used for their bacteriostatic properties. However, tetracycline analogs additionally possess nonantimicrobial properties, namely the ability to inhibit MMP activity. Because of these host-modulatory effects, doxycycline has been used for the treatment of inflammatory diseases such as osteoarthritis and refractory corneal ulcers in dogs.18–20 The effects of topical doxycycline application for the treatment of periodontitis have been investigated, and a commercial preparation is available for use in dogs.8,9 However, the method might be a 1-time remedy administered with dogs anesthetized, with the effects lasting only several days or weeks.2,8,9 Furthermore, susceptibility of various microbes to antimicrobials could change with a long-term systemic administration of antimicrobial doses of doxycycline.

Studies have demonstrated the benefit of SDDs as a systemic host-modulating treatment of human patients with periodontal disease. The SDD for humans is 20 mg/person every 12 hours; this dosage does not exert a subgingival antimicrobial effect and does not lead to changes in antimicrobial susceptibility among microflora, even during long-term use.22,23 The SDD in humans is approximately a fifth of the general antimicrobial dose and was experimentally confirmed to yield serum doxycycline concentrations of 0.6 to 0.8 µg/mL, which are considerably lower than the MIC of subgingival microflora as determined in vitro.22,23 Additionally, the genera of canine subgingival pathogenic anaerobes are similar to those of humans (eg, Porphyromonas spp, Bacteroides spp, and Prevotella spp), although the specific bacterial species differ.24–26 Findings of the present study showed the mean serum doxycycline concentration of dogs that received 1 or 2 mg of doxycycline/kg for 30 days was maintained at considerably < 1 µg/mL, which is the MIC for subgingival microflora.

Research concerning the pharmacokinetics of doxycycline polyphosphate in dogs shows that the maximum concentration time and the half-life of orally administrated doxycycline is 2.8 and 11.8 hours, respectively.27 The maximum concentration time was similar to that of the present study; however, the half-life tended to be extended to > 12 hours in all groups. Therefore, serum doxycycline concentration could exceed the MIC through periodic twice-daily administration even in dogs treated with 2 mg/kg/d. In general, gelatinolytic activity could not be maintained consistently in the once-daily dosing accompanied with the diurnal variation of serum doxycycline concentration. However, the serum concentration should remain below the MIC for the clinical use of SDDs in dogs to treat periodontitis, even though long-term medication might be continued.

Table 3—Mean ± SD gelatinolytic intensities and relative variations over 4 weeks in the dogs represented in Table 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>1 mg/kg/d</th>
<th>2 mg/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 (92 kDa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0 (AU)</td>
<td>162,908.02 ± 96,988.48</td>
<td>105,017.01 ± 89,518.88</td>
<td>150,371.73 ± 60,799.43</td>
</tr>
<tr>
<td>Variation in zymographic intensity (fold change)</td>
<td>1.29 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.85 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMP-2 (72 kDa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0 (AU)</td>
<td>96,676.43 ± 35,103.62</td>
<td>59,774.34 ± 32,858.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62,780.69 ± 35,243.54</td>
</tr>
<tr>
<td>Week 4 (AU)</td>
<td>124,537.10 ± 66,853.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41,648.80 ± 32,858.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42,457.11 ± 56,361.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Variation in zymographic intensity (fold change)</td>
<td>1.31 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.66 ± 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Across each row, values with different superscript letters are significantly (P < 0.05) different.

AU = Arbitrary units in the densitometer.
The expression of both pro-MMP-9 and active MMP-9 was greater in the control group, whereas dogs treated with doxycycline at 1 and 2 mg/kg/d had decreases in the activated form of MMP-9 in addition to reduction in the pro form of MMP-9. These results suggest that the SDD we identified reduces the expression of pro–MMP-9 but also might inhibit the conversion of the pro form into the activated form in dogs. Although zymography revealed no significant difference, the values of clinical variables of dogs treated with doxycycline at 2 mg/kg/d were significantly different from those of the other groups after the medication period.

Considering these results, a regimen of 2 mg of doxycycline/kg once daily could be used for the clinical improvement of periodontal disease in dogs, given that it yielded MMP-2 and -9 inhibition without an antimicrobial effect. The SDD could be recommended for long-term treatment of gelatinolytic inflammatory diseases such as periodontitis and arthritis. Because removal of injurious bacteria should be performed prior to the treatment for periodontitis, the medication protocol of SDD treatment should include subgingival scaling before SDD administration. Before clinical application of SDDs in dogs, additional in vivo studies, including evaluation of other biomarkers such as other MMPs, tissue inhibitor of metalloproteinase-1, and interleukin-6 that might support clinical improvement, but also including researches of antimicrobial resistance, would be needed for long-term use.

### References


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b. SST Tube, BD, Franklin Lakes, NJ.
c. Mallinckrodt Baker Inc, Phillipsburg, NJ.
d. Yakuri Pure Chemicals Co Ltd, Osaka, Japan.
e. Waters Co, Milford, Mass.
f. Series 200 LC, PerkinElmer Inc, Shelton, Conn.
g. TotalChrom Workstation, version 6.3.1, PerkinElmer Inc, Shelton, Conn.
h. Alltima C18 5 m, Grace Davison Discovery Science, Deerfield, Ill.
i. XP23-W Williams Explorer-Probe, Osung, Gimpo, Korea.
j. Tissue Extraction Reagent I, Invitrogen, Camarillo, Calif.
k. Protease Inhibitor Cocktail III, GenDEPOT, Barke, Tex.
l. Novex Tris-Glycine SDS Sample Buffer, Invitrogen, Carlsbad, Calif.
m. Novex Zymogram Renaturing Buffer, Invitrogen, Carlsbad, Calif.
n. Novex Zymogram Developing Buffer, Invitrogen, Carlsbad, Calif.
o. Amresco, Solon, Ohio.
p. Xpert Prestained Protein Marker, GenDEPOT, Barke, Tex.
q. Multi-gauge, version 3.0, Fujifilm, Tokyo, Japan.
r. LAS-3000, Fujifilim, Tokyo, Japan.
s. PASW Statistics, version 18, SPSS Inc, Chicago, Ill.


