A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Evaluation of Subantimicrobial Dose of Oral Doxycycline for the Treatment of Periodontitis in Dogs

개에서 치주염 치료를 위한 항생농도 이하 doxycycline의 경구투여에 대한 평가

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ABSTRACT

Periodontal inflammatory disease is one of the most prevalent disease affecting both humans and small animals. Prior to any other treatments for the chronic periodontitis, mechanical removal of plaque and calculus should be achieved. In human medicine, several medical treatment options were established after the periodontal debridement. Subantimicrobial dose of doxycycline (SDD) is one of adjunctive treatments which downregulate the activity of matrix metalloproteinases (MMPs), which are key destructive enzymes in periodontitis in humans. Therefore, the purpose of the present study was to evaluate the SDD of dogs which would not reach the antimicrobial concentration, but had the inhibitory effect of matrix

metalloproteinases using experimentally induced periodontitis model. This study consists of three chapters.

Chapter I demonstrated the effectiveness of modified silk ligature twisted with wire for the induction of advanced periodontitis. Twenty beagles were divided into 4 groups according to the ligature-inducing materials: soft-moistened food only, wire ligature, silk ligature and silk-wire twisted ligature. Experimental periodontitis was induced by ligation using each material according to the groups on the cervix of 6 teeth in each dog. The periodontal inflammation status was evaluated using clinical periodontal parameters and dental radiography before and 60 days after ligation. The ligatures were checked daily, and the day the ligature fell out was recorded. The silk-wire twisted ligature induced the most intense periodontitis and remained the most stable compared to the other groups ($P<0.05$).

Chapter II demonstrated the identification of SDD for the treatment of periodontitis in dog. Twenty healthy beagles were used for measurement of the serum concentration of doxycycline. The beagles were divided into 4 groups according to the administration dose. Doxycycline hyclate was orally given at a single dose of 1, 2, 3 and 5 mg/kg. Blood samples were collected pre- and post-administration at the same time and the serum concentrations of doxycycline were determined using high-performance liquid chromatography. Mean serum doxycycline concentrations of 1 and 2 mg/kg groups were maintained significantly lower than the minimal inhibitory concentration of doxycycline during 24 hours. The evaluated SDD was used for the assessment of the matrix metalloproteinase inhibitory effects using gelatin zymography. Fifteen beagles with periodontitis were used for the evaluation of the efficacy of SDD for one-month administration.

Five beagles were assigned to each group, and each group was orally administrated 0, 1 and 2 mg/kg of doxycycline once a day. Before and after the experimental phase, clinical periodontal status and MMPs expression of periodontium were evaluated. The zymographic intensities which reflected MMPs expression significantly decreased in the SDD administrated groups compared to control group. Clinical improvements of periodontal status were dose-dependent ($P<0.05$).

Chapter III demonstrated the influence of oral administration of SDD to the normal periodontal flora. Experimental periodontitis using the silk-wire twisted ligature were induced in twelve healthy beagles. The dogs were randomly assigned to one of two groups: SDD (doxycycline, 2 mg/kg) and control group. Clinical periodontal status was evaluated and subgingival plaque was sampled on weeks 0, 4 and 8. Sampled plaque was cultured in the completely anaerobic system for one week and the total anaerobes, *Porphyromonas* spp., *Bacteroid* spp. and *Pasturella* spp. were counted. Using the agar dilution method, minimum bactericidal concentration of doxycycline was evaluated and resistance for doxycycline was monitored during experimental phase. The clinical periodontal status of SDD group was significantly improved compared to the control group. All kinds of bacterial counts were not significantly different between the two groups. Antibacterial resistance was not established in the SDD group during the experimental periods ($P<0.05$).

Based on the results of the present studies, oral administration of 2 mg/kg doxycycline, once a day, was identified as appropriate dose for the periodontitis which should not affect the antimicrobial susceptibility of the periodontal flora in the dog. Therefore, it is suggested that once a day oral regimen of 2 mg/kg

doxycycline could serve as a SDD for periodontitis in dogs.

Keywords: subantimicrobial dose, doxycycline, periodontitis, dog

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CHAPTER II.

Determination of Subantimicrobial Dose of Doxycycline for the Treatment of Periodontitis in Beagle Dogs

CHAPTER III.

Clinical and Microbiological Effects of Subantimicrobial Dose of Oral Doxycycline on Periodontitis in Dogs

GENERAL INTRODUCTION

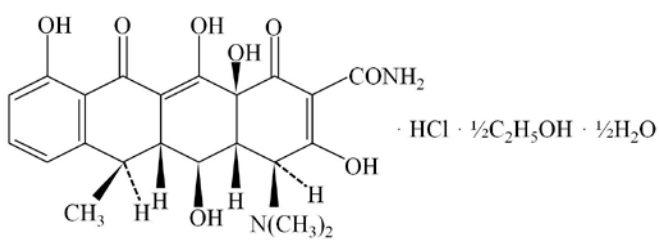
Periodontium is the supportive structure of teeth which include gingiva, cementum, periodontal ligament and alveolar bone (Bellows, 2004). Periodontitis is the inflammation involving a destruction of periodontal structures which is caused by host's immune response against bacterial by-products (Bellows, 2004). It is the most common oral disease in dogs over the age of three years (Gorrel, 2004; Hamp *et al.*, 1984). Calculus is caused by the accumulation of minerals and bacterial by-products on the teeth. It maintains and accelerates periodontal disease by keeping plaque close to the gingival tissue in addition to decreasing the possible area for repair and re-attachment of periodontium (Bellows, 2004). Therefore, a mechanical removal of plaque and calculus should be achieved prior to any other medical intervention for the treatment of periodontitis (Bellows, 2004; Gorrel, 2004).

In human medicine, several researches revealed pharmacological modulation of exaggerated host inflammatory and immune responses in addition to mechanical elimination of microbes were beneficial in the treatment of periodontitis (Gurkan *et al.*, 2005; Salvi and Lang, 2005). The matrix metalloproteinases (MMPs), a family of host derived enzymes, are intimately involved in degradation of extracellular matrix during both normal tissue remodeling and pathologic conditions (Ramamurthy *et al.*, 2002). Excessive activity of MMPs is widely recognized a hallmark of both experimental and naturally-occurring human periodontal disease, leading to the loss of gingival collagen and destruction of alveolar bone (Ramamurthy *et al.*, 2002).

Tetracyclines had been identified as MMPs inhibitors in the early 1980 (Golub

et al., 1983; Golub *et al.*, 1985; Ramamurthy and Golub, 1983). Both human and veterinary dentistry, topical administrations of the tetracyclines to the subgingival space is identified as effective therapy for the periodontitis when used as an adjunct to mechanical debridement (Hayashi *et al.*, 1998; Hirasawa *et al.*, 2000; Vandekerckhove *et al.*, 1998; Wennström *et al.*, 2001; Zetner and Rothmueller, 2002). For the long-term management of periodontitis, repeated applications of the tetracycline ointments are beneficial after a single episode of supra- and subgingival scaling because the effect of the tetracyclines last for a short period (Dannewitz *et al.*, 2009; Hirasawa *et al.*, 2000). However, the repeated application to the subgingival space is not a simple method in veterinary medicine because each application requires general anesthesia in the canine patients.

For the treatment of human periodontitis, doxycycline, a kind of tetracyclines, may also administer orally in the long term to last the continuous MMPs inhibitory effect (Caton, 1999). Doxycycline is a semi-synthetic bacteriostatic antibiotic which derived from oxytetracycline. Doxycycline is available as several forms, and among them, the hyclate salt is generally used for the treatment of human periodontitis (Caton, 1999; Plumb, 2004). Doxycycline hyclate has the following empirical formula: $(C_{22}H_{24}N_2O_8 \cdot HCl) \cdot \frac{1}{2}C_2H_6O \cdot \frac{1}{2}H_2O$.



The antibiosis of doxycycline is accomplished through inhibiting protein synthesis by reversible binding to 30S ribosomal subunits of susceptible organisms,

thereby preventing binding to those ribosomes of aminoacyl transfer-RNA (Plumb, 2004). Compared to the other tetracyclines, doxycycline is the most suitable for the oral administration due to its greater lipid solubility, which can account for superior absorption and distribution, and prolong the half-life (Castro *et al*., 2009; Wilson *et al*., 1988). The variation in half-life is dependent on the route of administration, or chemical formulation (Wilson *et al*., 1988). In some animal species, per oral administration of doxycycline is not able to reach as high as the serum level of intravenous injection, but has more delayed half-life compared to the intravenous injection of the same dose doxycycline (Castro *et al*., 2009; Michel *et al*., 1979; Wilson *et al*., 1988).

It is previously revealed that the MMPs inhibitory effect of tetracyclines is independent of their antimicrobial activity (Golub *et al*., 1991). Therefore, subantimicrobial dose of doxycycline (SDD) has been used as one of adjunctive treatment which downregulated the activity of MMPs in humans (Choi *et al*., 2004; Emingil *et al*., 2004a; Lee *et al*., 2004). The long-term oral administration of SDD does not exert antibacterial effect and lead to the changes in antimicrobial susceptibility which was confirmed as clinically effective in human periodontitis (Caton and Ryan, 2011; Thomas *et al*., 2000; Walker *et al*., 2000).

In parallel to humans, it could be postulated that SDD was an effective adjunctive method to the periodontal treatment in dogs. To evaluate the effect of SDD objectively on periodontitis in dogs, the experimental procedure has to use a standardized periodontitis model. Experimental periodontitis has been raised by acceleration of accumulating plaque into the periodontal pocket in which the ligature induced periodontitis (de Oliveira *et al*., 2011; Ramamurthy *et al*., 2002;

Tubb *et al*., 1990). In general, the experimental models of ligature induced periodontitis used hygroscopic and braided-type ligatures such as silk (de Oliveira *et al*., 2011; Ramamurthy *et al*., 2002). However, invasive interference has to be involved to maintain the silk ligature to the tooth cervix because of the physical characteristics in which the tensile strength reduction (Fossum, 2002; Tubb *et al*., 1990). In addition, the loosening and lost ligature acts as undesired variables which is not able to provide sufficient periodontitis inducement. Therefore, more stable and plaque-accumulating ligature should be used for the standardized periodontitis induction in the experimental study.

The purpose of this study was to identify more effective methods for periodontitis induction using modified ligatures (Chapter I), to evaluate the optimal dose of doxycycline not reaching the antimicrobial concentration but sustaining the inhibitory effect of matrix metalloproteinases (Chapter II), and to evaluate the clinical and microbiological effect of SDD using the standardized periodontitis model in dogs (Chapter III).

CHAPTER I.

A Modified Method for Inducing Periodontitis in Dogs Using a Silk-Wire Twisted Ligature

Abstract

This study was designed to assess the effectiveness of a modified silk ligature twisted with wire for inducing advanced periodontitis. Periodontitis was induced in five premolars and one molar of 20 healthy dogs over a 60-day period. The dogs were divided into four groups according to the ligature materials used: soft moistened food only (SF), wire ligature (WL), silk ligature (SL), and twisted ligature with silk and wire (SWL). Periodontal indices were recorded, and dental radiographs were taken before and after 60 days of ligation. The ligatures were checked daily and the day of the ligature fell out was noted. The period during which the ligatures were maintained was significantly shorter for the SL group compared to the SWL group ($P<0.05$). Results of the clinical examination showed that almost all periodontal status parameters including the plaque index, gingival index, clinical attachment level, and bleeding on probing were significantly exacerbated in the SWL group compared to the other groups ($P<0.05$). Radiographic evaluation demonstrated that alveolar bone levels were significantly lower in the SWL group than the other groups on day 60 ($P<0.05$). These results suggested that experimental periodontitis induced by SWL could be an effective method for investigating periodontitis in canine models.

Introduction

Periodontal inflammatory disease is one of the most prevalent diseases affecting both humans and small animals (Bellows, 2004; Haze *et al.*, 2009). Therefore, several studies have examined the occurrence and treatment of periodontal disease using experimentally induced periodontitis models This condition has been induced in rats using a range of methods in which pathogen intake, endotoxin injection, high carbohydrate feeding, or ligature placement around the tooth cervix (Okada *et al.*, 2010; Ramamurthy *et al.*, 2002; Tomofuji *et al.*, 2006; Xie *et al.*, 2011). Compared to the rat model, the ligature-induced periodontitis model was used more frequently in dogs (de Oliveira *et al.*, 2011; Tillmanns *et al.*, 1998; Zitzmann *et al.*, 2004).

Ligation materials used to induce periodontitis including silk, cotton, and nylon are placed between the tooth and periodontium to mechanically widen the periodontal pocket and to facilitate plaque accumulation in the dento-gingival region (Ericsson *et al.*, 1975). Several studies have induced experimental periodontitis using a more invasive method in which a full-thickness gingival flap is made and the alveolar bone is destroyed prior to ligation (de Oliveira *et al.*, 2011; Tubb *et al.*, 1990). These methods take several months to promote advanced periodontitis, and thread-type ligatures might be lost due to loosening or wearing during this period. In general, ligatures that are lost during the periodontitis induction phase are immediately replaced to maintain the same induction period and environment (de Oliveira *et al.*, 2011; Ramamurthy *et al.*, 2002). These reparative procedures required redundant general anesthesia. Furthermore, the induction site can be affected by the additional manipulations that may act as

variables in the experimental prcedure. Therefore, present study was designed to identify more effective methods for periodontitis induction using modified ligatures, which can promote cases of more advanced periodontitis using a less invasive approach and reduce unnecessary interventions compared to previously established techniques.

Materials and Methods

The protocol of this study was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-100208-3). Twenty beagle dogs (13 females and 7 males) approximately 1.5 years old were used in this study. All experimental operations and examinations were performed with dogs under general anesthesia induced by a combination of medetomidine (0.01 mg/kg; Domitor®, Orion Pharma, Espoo, Finland), tramadol (2 mg/kg; Toranzin inj., Samsung Pharm. Ind. Co., Ltd., Seoul, Korea), and commercial combination of zolazepam and tiletamine (2.5 mg/kg; Zoletil® 50, Virbac Laboratories, Carros, France) administered via intramuscular injection. To prepare the healthy gingiva, all teeth were scaled and polished using a piezoelectric ultrasonic scaler (ART-SP2, Bonart Co., Ltd, La Puente, USA), and tooth brushing was performed once a day without anesthesia for the following 2 weeks. During these periods of prophylactic care, the beagles were fed a hard pellet diet to reduce plaque formation (Martuscelli *et al*., 2000).

1. Experimentally induced periodontitis

Two weeks after scaling, experimental periodontitis was induced on the left maxillary second premolar (PM2), third premolar (PM3), and fourth premolar (PM4) as well as the left mandibular PM3, PM4, and first molar (M1). Prior to ligation, the gingival attachment was incised slightly by inserting a number 11 scalpel blade (Stainless steel surgical blade No. 11, Ailee Co., Ltd., Busan, Korea), and the periodontal ligaments were undermined until a periodontal pocket depth was reached up to 3 mm with a straight elevator (E301, Osung MND. Co., Ltd., Gimpo, Korea). After undermining, shallow notches for ligature retention were made in the mesial and distal cervical region of each tooth with a # 1 round bur (Komet® Carbide bur RA 1, Komet® USA, Rock-Hill, USA). An ancillary notch was placed in the mesial surface of the maxillary PM4.

The dogs were randomly assigned to four groups. Periodontitis was induced in three groups (Fig. 1) by tying the ligations around the cervical region of the tooth using 0.012 inch dental ligature wire [wire ligature (WL); n=5; teeth=30; Spooled ligature wire, ClassOne Orthodontics, Lubbock, USA], 2-0 braided silk [silk ligature (SL); n=5; teeth=30; Silk 2-0, Ailee Co., Ltd., Busan, Korea] or a twisted wire with 2-0 silk [silk-wire twisted ligature (SWL); n=5; teeth=30]. The remaining group did not undergo any surgical intervention but was fed only soft-moistened food as a control (SF; n=5; teeth=30). For pain control, tramadol (4 mg/kg, intramuscular injection) was administered twice a day for 3 days after placing the ligatures. To promote plaque formation, soft-moistened food

was given to all groups for the following 60 days. The ligatures were checked daily, and the day the ligature fell out was recorded.

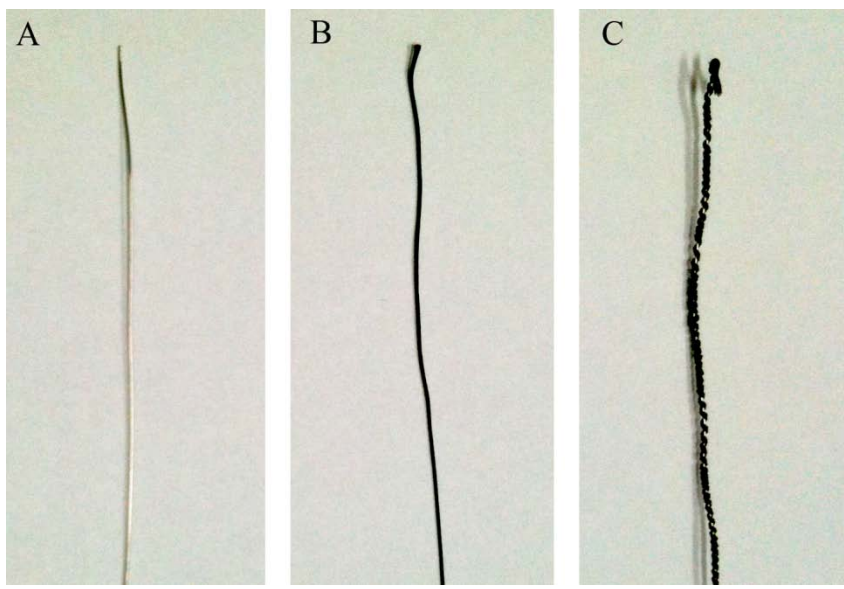


Fig. 1. Ligature materials. A. 0.012 inch dental ligature wire, B. 2-0 braided silk, C. Twisted ligature with silk and wire.

2. Periodontal status evaluation

Before (Day 0) and 60 days after (Day 60) ligature placement, the clinical periodontal parameters were recorded and digital dental radiographs were taken to evaluate the periodontal status. Clinical parameters (Fig. 2) included the plaque index (PI), gingival index (GI), periodontal pocket depth (PPD), clinical attachment level (CAL), and bleeding on probing (BoP) (Löe and Silness, 1963; Silness and Löe, 1964; Wennström *et al.*, 2001). All measurements were taken at three sites per tooth. If a ligature fell out, the parameters were measured on that day. All measurements were taken by one experienced clinician using a Williams periodontal probe (XP23-W Williams Explorer-Probe, Osung MND, Gimpo, Korea).

Digital intraoral radiographs (Image-Vet 70® ACP, AFP Imaging Corporation, Elmsford, USA) were obtained to evaluate the amount of alveolar bone loss after ligature application. An intraoral sensor (EVA-Vet size #2, AFP Imaging Corporation, Elmsford, USA) was positioned to take dental radiographs using bisecting and parallel technique for the maxillary and mandibular teeth, respectively, with the same exposure protocol. The alveolar bone level (ABL) was measured using a software program (Photoshop® CS5, Adobe® Systems Inc. San Jose, USA). This measurement was performed at the mesial and distal margin of each tooth except for the maxillary PM4. ABL of maxillary PM4 was only measured at the distal margin of the tooth because the ABL at the distal margin was difficult to distinguish from the other superimposed dental structures such

as the maxillary M1. Distances from alveolar bone margin and cemento-enamel junction to the root apex were measured, and the ABL was calculated as a ratio of these two lengths.

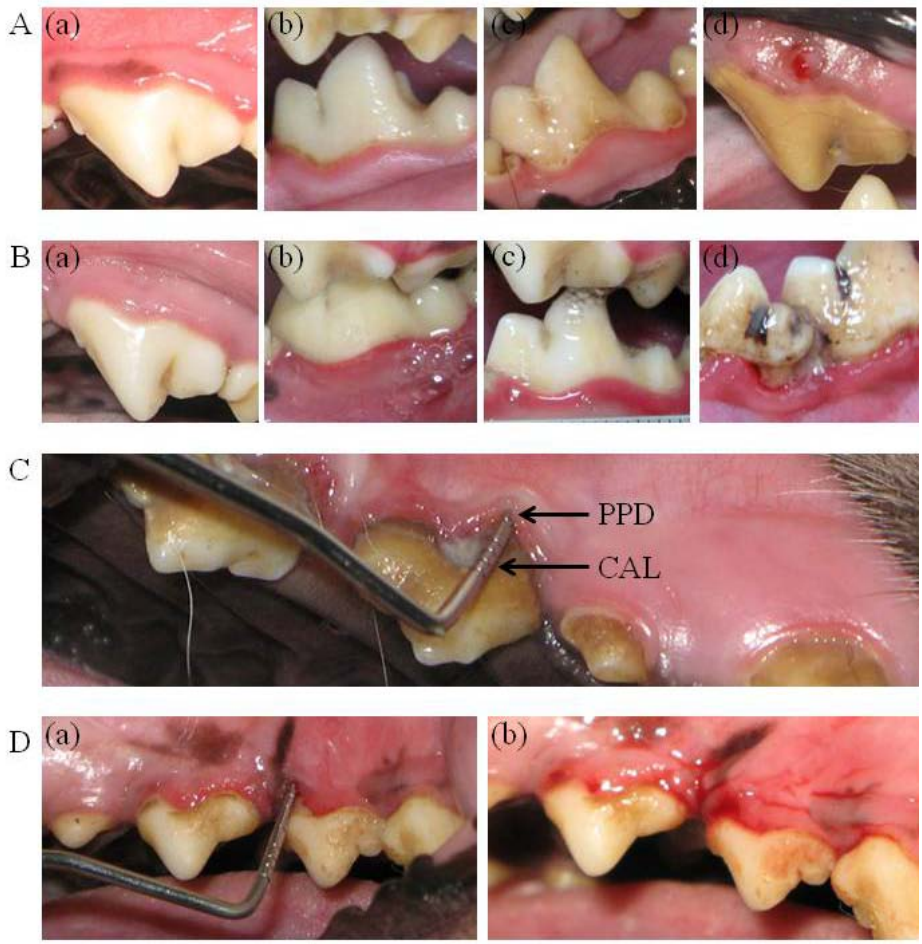


Fig. 2. Representative features of periodontal parameters. A. Plaque index (PI), (a) PI 0: no plaque, (b) PI 1: a film of plaque adhering to the free gingival margin and adjacent area of the tooth (not more than 1mm), (c) PI 2: moderate accumulation of soft deposits within the gingival pocket, or on the tooth and gingival margin (less than one half of crown), (d) PI 3: abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin (more than one half of crown), B. Gingival index (GI), (a) GI 0: absence of gingival inflammation, (b) GI 1: mild gingival inflammation - slight change in color of gingival margin and little change

in texture, (c) GI 2: moderate gingival inflammation – moderate glazing, redness, edema, hypertrophy, and/or bleeding on pressure, (d) GI 3: severe gingival inflammation- marked redness and hypertrophy, spontaneous bleeding and ulceration, C. Periodontal pocket depth (PPD): the diatance between the gingival margin and the bottom of the probeable pocket; Clinical attachment level (CAL): the distance between the cement-enamel junction and the bottom of the probeable pocket, D. Bleeding on probing (BoP): Probing (a) which was followed by no bleeding referred to BoP 0 (b) whereas bleeding was marked BoP 1.

3. Statistical analyses

All data was analyzed statistically using a software program (SPSS 12.0, SPSS Inc., Chicago, USA). The maintenance period and clinical parameters for each group were expressed as the mean ± standard deviation (SD). Differences in the maintenance periods between the SWL and WL or SL groups were assessed using a Student's *t*-test. The clinical parameters measured on day 0 and day 60 in the same group were analyzed using a paired *t*-test to compare the progression of periodontitis in the same tooth within each group. A one-way analysis of the variance (ANOVA) was used to perform an intergroup comparison of the clinical periodontal parameters and ABL change on days 0 and 60 with Tukey method as a *post hoc* test. Data within the 95% confidence level were considered significant.

Results

1. Ligature maintenance periods

The maintenance periods and the maintaining ratio were compared according to ligature materials (Table 1). The SF group was excluded from this analysis because the teeth in these animals were not tied with any material. Among the 30 teeth in each group, 26 and 25 ligatures were maintained for 60 days in the WL and SL groups, respectively. All ligatures were retained by the SWL group during the entire experimental period. Among the teeth that did not retain the ligature, most were maxillary PM4 (n=6; 66.6%). The mean ligature maintenance period was significantly shorter for the SL group than the SWL group (P=0.046). Although the ligature maintenance period for the WL group was also short compared to the SWL group, this difference was not significant.

Table 1. Ligature maintenance periods and the ratio of ligature loss

Group	Ratio of ligature loss (n / N)	Ligature maintenance period	
		days*	P-value†
WL	4 / 30	54.7 ± 14.6	0.051
SL	5 / 30	53.2 ± 16.2	0.046
SWL	0 / 30	60.0 ± 0.0	1.000

*Data are expressed as the mean ± SD. †Statistical significance relative to SWL group. WL: wire ligature, SL: silk ligature, SWL: silk-wire twisted ligature, n: the number of ligatures that fell out for the group, N: the total number of teeth with ligatures in the group.

2. Periodontal status

The mean and SD values for the clinical periodontal parameters on days 0 and 60 were presented (Table 2). Baselines for the clinical parameters of the periodontal status and ABL among the study groups were not significantly different on day 0. After the 60 day period of periodontitis induction, significant increases in all periodontal parameters were observed for the WL, SL and SWL groups compared to day 0 ($P<0.05$; Fig. 3). On the other hand, only the PI, GI and PPD increased significantly in the SF group ($P<0.05$).

In the intergroup comparison of GI, CAL and BoP, significant differences were observed among each experimental group, and these parameters were increased in a similar manner up to day 60 in each experimental group (Table 2). SWL animals showed the most dramatic changes followed by the SL and WL groups ($P<0.05$). The SF group had the best GI values compared to the other groups ($P<0.05$) with no significant increases in the CAL and BoP compared to baseline values. The mean PI scores of the SF and WL groups on day 60 were not significantly increased compared to that of the SL group, which was significantly lower than the SWL group score ($P<0.05$). In addition, the mean PPD on day 60 was significantly lower in the SF and WL groups than the SL and SWL groups ($P<0.05$).

ABL of each experimental group was presented (Fig. 4) and the variations of ABL were compared between the groups (Fig. 5). The ABL of the SF group did not significantly change from days 0 to 60. On the other

hand, the WL, SL and SWL groups showed significantly lower ABLs on Day 60 compared to day 0 ($P<0.05$). The ABLs of each group were significantly different on day 60; the SWL group had the lowest ABL followed by the SL, WL and SF groups ($P<0.05$).

Table 2. Clinical periodontal parameters

Day	Parameter	SF	WL	SL	SWL
Day 0	PI	0.3 ± 0.5	0.2 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
	GI	0.4 ± 0.7	0.3 ± 0.7	0.2 ± 0.6	0.2 ± 0.6
	PPD	1.25 ± 0.41	1.32 ± 0.41	1.37 ± 0.45	1.33 ± 0.46
	CAL	0.08 ± 0.27	0.02 ± 0.15	0.06 ± 0.23	0.04 ± 0.21
	BoP	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
Day 60	PI	$1.9 \pm 0.8^{a,*}$	$2.0 \pm 0.7^{a,*}$	$2.3 \pm 0.7^{b,*}$	$2.9 \pm 0.4^{c,*}$
	GI	$0.9 \pm 0.7^{a,*}$	$1.9 \pm 0.6^{b,*}$	$2.1 \pm 0.6^{c,*}$	$2.8 \pm 0.4^{d,*}$
	PPD	$1.64 \pm 0.52^{a,*}$	$1.83 \pm 0.55^{a,*}$	$2.07 \pm 0.85^{b,*}$	$2.13 \pm 0.79^{b,*}$
	CAL	0.10 ± 0.40^{a}	$0.97 \pm 0.57^{b,*}$	$1.91 \pm 0.77^{c,*}$	$2.41 \pm 0.67^{d,*}$
	BoP	0.2 ± 0.4^{a}	$0.6 \pm 0.5^{b,*}$	$0.7 \pm 0.5^{c,*}$	$0.9 \pm 0.3^{d,*}$

Data are expressed as the mean ± SD (n=5; 30 teeth). ^{a,b,c,d}Values with different superscript letters across each row are significantly different. *Significantly increased values within a single group compared to the same parameters measured on day 0. PI: plaque index, GI: gingival index, PPD: periodontal pocket depth, CAL: clinical attachment level, BoP: bleeding on probing, SF: control, WL: wire ligature, SL: silk ligature, SWL: silk-wire twisted ligature.

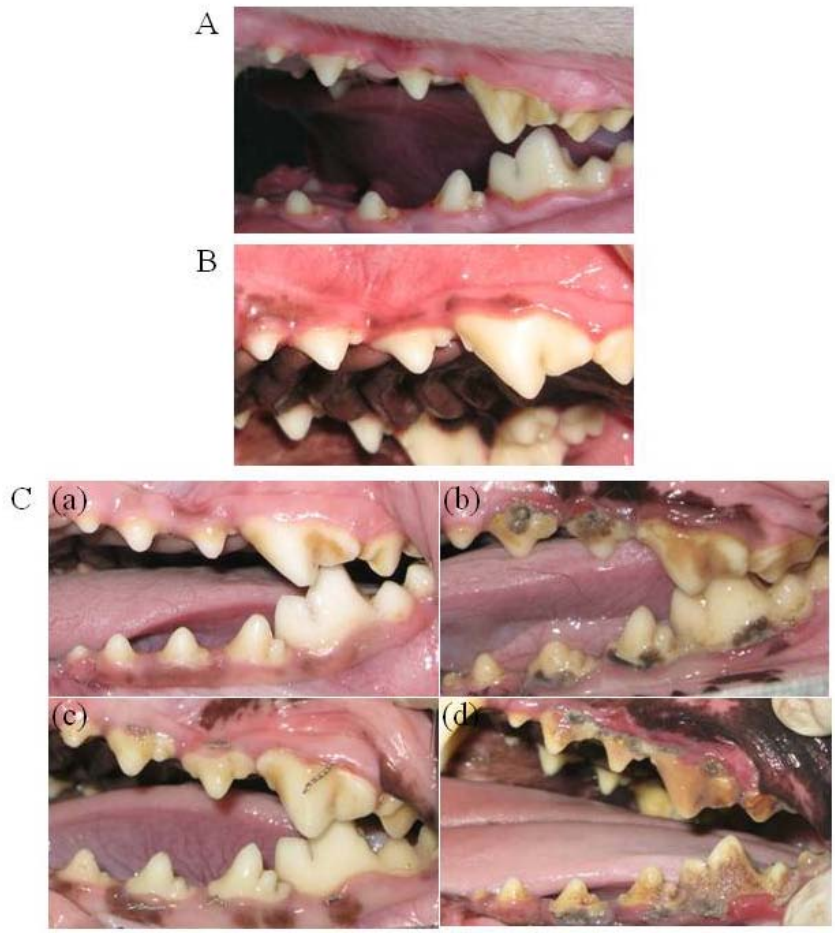


Fig. 3. Periodontal status of before and after periodontitis induction. A. Before initial dental prophylaxis, B. After initial dental prophylaxis, C. 60 days after the periodontitis induction, (a) SF group (soft-moistened food only), (b) SL group (2-0 silk ligature), (c) WL group (0.012 inch dental wire ligature), (d) SWL group (silk-wire twisted ligature).

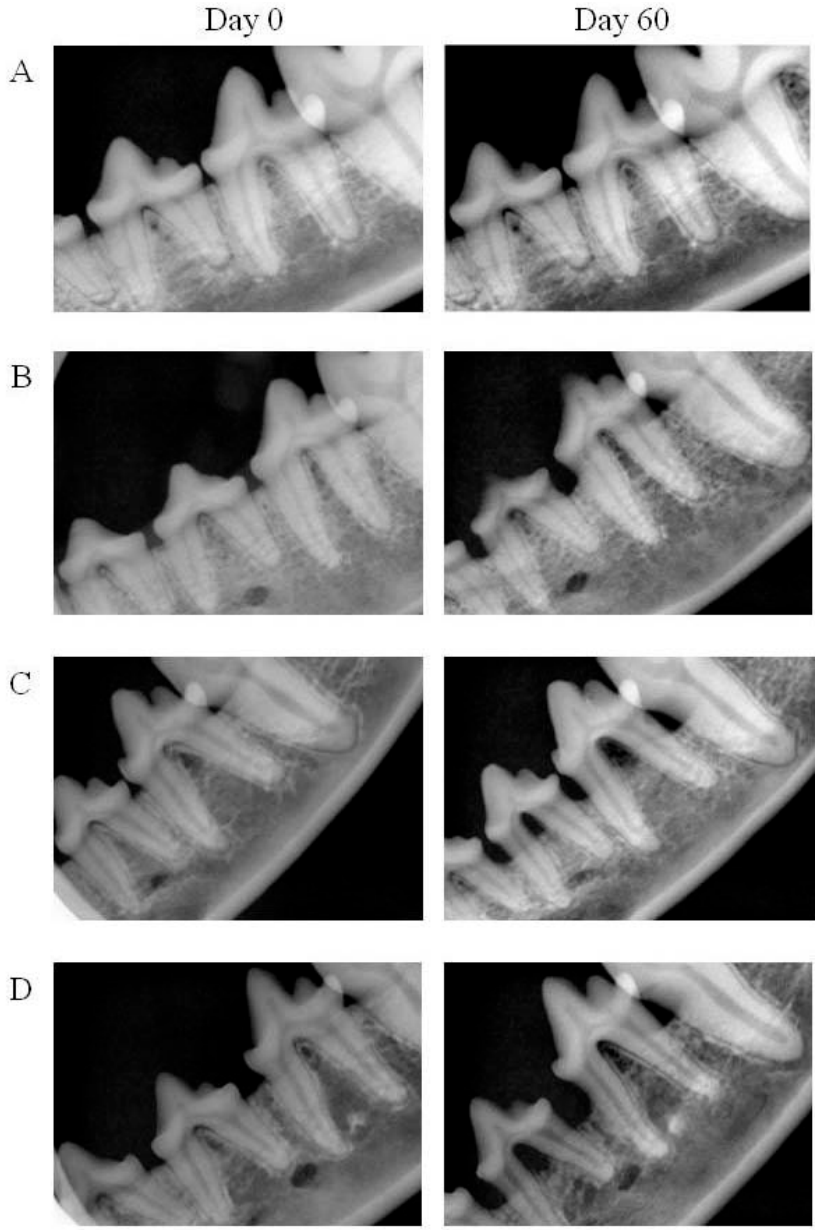


Fig. 4. Representative dental radiography of alveolar bone levels at the days 0 and 60. A. SF group (soft-moistened food only), B. SL group (2-0 silk ligature), C. WL group (0.012 inch dental wire ligature), D. SWL group (silk-wire twisted ligature).

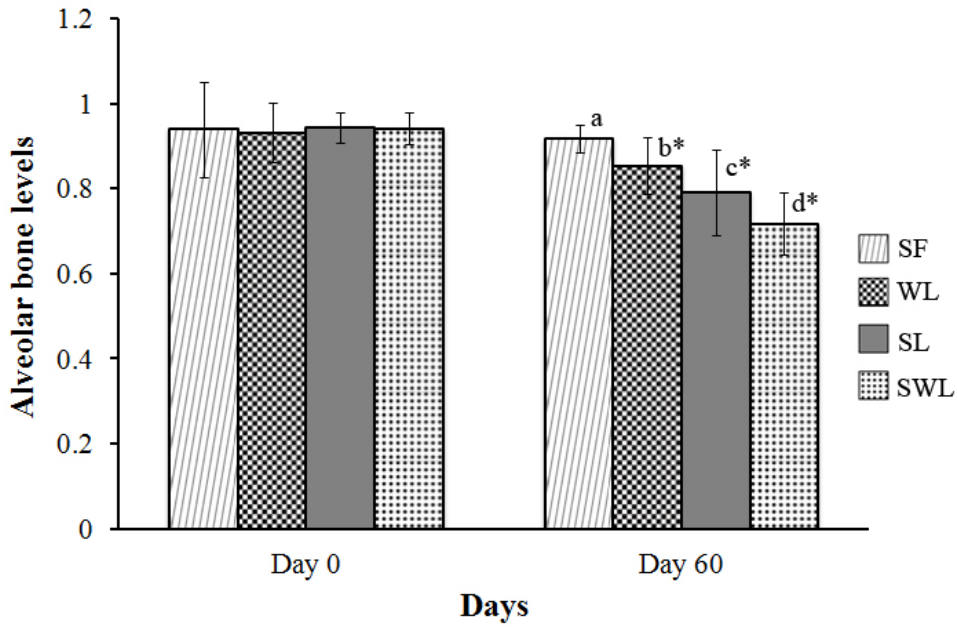


Fig. 5. Alveolar bone levels before (day 0) and 60 days later (day 60) the periodontitis induction using ligatures.

[a,b,c,d]Values with different superscripts are significantly different at same day. [*]Significantly different compared to day 0 in same group. SF: control, WL: wire ligature, SL: silk ligature, SWL: silk-wire twisted ligature.

Discussion

Periodontitis is a chronic inflammatory reaction that occurs as a consequence of interactions between the host immune system and oral pathogens (Kirkwood *et al.*, 2007; Salvi and Lang, 2005). Therefore, previous experimental studies investigating periodontal disease have generally used the animal models of periodontitis gradually induced by promoting plaque accumulation (de Oliveira *et al.*, 2011; Ericsson *et al.*, 1975; 1987; Lindhe *et al.*, 1973; Martuscelli *et al.*, 2000; Schroeder and Lindhe, 1975; Tubb *et al.*, 1990). The model most similar to naturally occurring cases of periodontitis is one in which this condition is induced by a carbonate-enriched diet consisting of soft food. Nevertheless, the induction period for this method is greater than 5 to 7 month in dogs after clinical gingivitis has developed (Lindhe *et al.*, 1973). In this study, the control group (SF) showed no significant changes in CAL or BoP (factors that can represent destructive periodontal alterations) up to 60 days after the initial SF feeding.

Compared to the previous studies, it was found that the use of hygroscoic and braded-type ligatures to promote periodontitis had a shorter induction period in dogs. In some studies, cotton floss with or without wire, which can provoke significant tissue reactions within the periodontal tissue, had been used to induce periodontitis (Ericsson *et al.*, 1975; 1987; Schroeder and Lindhe, 1975; Tillmanns *et al.*, 1998; Tubb *et al.*, 1990). In contrast to cotton, the degree of tissue reaction to a SL is quite low whereas wire has been shown to produce no reaction (Castelli *et al.*, 1978; Fossum, 2002). Although tissue reactions resulting from cotton do not directly cause an inflammatory response, the present study used silk with wire ligatures to reduce unnecessary environmental changes caused by the ligature.

For ligature-induced periodontitis, the ligature material not only forms a periodontal pocket subject to plaque accumulation but also serves as a constant irritant that can harbor a multitude of oral pathogens (Tubb *et al.*, 1990). Considering these characteristics, the loss of ligatures that enhance plaque formation might result in not induce experimental periodontitis. For these reasons, other groups performed studies in which shallow notches were created on the mesial and distal cervix of the tooth using a round bur to act as retentive grooves for the ligature (de Oliveira *et al.*, 2011; Ramamurthy *et al*., 2002). Another study employed more invasive method in which a full thickness gingival flap was created to secure the ligature to the alveolar bone crest (Tubb *et al*., 1990). In the present study, retentive grooves were made at two points of each tooth cervix to fix the ligature onto the initial location without loosening whereas a gingival flap was not made. Instead of using a relatively invasive method, a closed undermining of the pocket was performed up to a depth of 3 mm to expose the cement-enamel junction in this study.

The period of ligature maintenance was significantly decreased in the SL group whereas all ligatures in the SWL group were maintained for 60 days. These results could be due in part by the fact that the tensile strength of silk sutures in tissue was reduced within 14 days and the relative security of the knot was also poor, whereas stainless steel ligatures were stable in the tissue (Fossum, 2002). The ligature maintenance period of the WL group also decreased compared to SWL group, although this difference was not significant. The smooth surface of the wire might have cause the ligature to slip and fall out because the wire had been loosened by cervical connective tissue breakdown in the WL group.

Differences in clinical periodontal status varied significantly according to the ligature materials used. The SL and SWL groups, in which ligatures containing silk were used, showed significantly more advanced periodontal destructive changes than the other groups. Furthermore, most periodontal indices, including PI, GI, CAL, BoP, and ABL, were significantly worsened in the SWL group compared to the SL group. Considering the ability of ligatures to harbor oral pathogens while inducing periodontitis as mentioned above (Tubb *et al*., 1990), these differences between the SL and SWL groups were attributed to the stability and surface area of silk. In the SWL group, the twisted ligature made with silk and wire could not only be firmly secured around the cervix of tooth due to the tension of the wire, but the coiling silk also provided a wide surface that housed a multitude of pathogenic micro-organisms.

Two cases of oral mucosal ulceration were encountered in both of the WL and SWL groups. The ligature used for both these animals included wires, and the ulceration was attributed to a twisted knot of wire. Nevertheless, oral ulceration was not observed in all animals with ligatures containing wire. These results indicate that care should be taken when making the knot so as not to directly disturb the oral mucosa.

Conclusions

In summary, it was found that ligatures composed of twisted silk and wire were maintained for a significantly longer time than ones made of silk in the present study. Furthermore, the SWL induced more significantly advanced cases of experimental periodontitis compared to the other types of ligatures over the same period of time. According to these results, twisted silk and wire ligatures can be an effective method for the canine periodontitis model.

CHAPTER II.

Determination of Subantimicrobial Dose of Doxycycline for the Treatment of Periodontitis in Beagle Dogs

Abstract

This study was designed to identify the subantimicrobial dose of doxycycline (SDD) for the treatment of periodontitis in dogs. Twenty healthy beagles were used for measurement of the serum concentration of doxycycline and were divided into 4 groups. Doxycycline hyclate was given orally at a single dose of 1 (Group 1), 2 (Group 2), 3 (Group 3) and 5 mg/kg (Group 4). Blood samples were collected pre- and post-administration at the same time and the serum concentrations of doxycycline were determined using high-performance liquid chromatography. The average of serum doxycycline concentration was calculated, and SDD was evaluated. For the evaluation of the efficacy of SDD, the other 15 beagles with periodontitis were used and were devided into 3 groups. The evaluated SDD (1 and 2 mg/kg) was given orally to each group (Groups B and C, respectively) once a day, and the control group (Group A) was fed vehicle only during the 1-month period. Clinical attachment level (CAL) and bleeding on probing (BoP) scores were recorded. Gingival samples were collected before and after the 1-month medication period from the same locations in dogs afflicted with periodontitis. Matrix metalloproteinase inhibitory effects of pre- and post-medication samples were compared using gelatin zymography. Mean doxycycline concentrations of Groups 1 and 2 were maintained significantly lower than the minimal inhibitory concentration of doxycycline during 24 hours. The zymographic intensities were significantly increased in Group A, while decreasing in Groups B and C relative to A , and the CAL and BoP scores were significantly improved in Group C compared to Groups A and B ($P<0.05$). According to the results of the present studies, it is

suggested that 2 mg/kg doxycycline once a day would be an appropriate SDD in dogs.

Introduction

Periodontal disease occurs due to multiple interactions between bacterial biofilms and inflammatory response of the periodontal connective tissue. Pathologically, several Matrix metalloproteinases (MMPs) play key roles in periodontal tissue breakdown by initiating extracellular matrix degradation (Kinane, 2000). Tetracyclines can have therapeutic value by improving periodontal status due to inhibition of MMPs, independent of their antimicrobial activity (Golub *et al*., 1991). Among them, Subantimicrobial dose of doxycycline (SDD) therapy has been intensively investigated for the treatment of chronic periodontitis in human medicine (Choi *et al*., 2004; Emingil *et al*., 2004a; 2004b; Golub *et al*., 1995). The subantimicrobial doxycycline plasma concentration is recommended to be below the minimum inhibitory concentration (MIC) of 1 µg/ml in humans (Caton, 1999).

In canine dentistry, the topical administration of doxycycline can resolve periodontal inflammation and improve periodontal status (Polson *et al*. 1996; Zetner and Rothmueller, 2002). A commercial topical doxycycline preparation in a polymer matrix can bind to the tooth surface. The subsequent slow release of doxycyclin from the polymer matrix exerts a therapeutic effect consisting of antibiosis and local inhibition of collagenase activity (Golub et al., 1991; Zetner and Rothmueller, 2002). However, to date, there has been no investigation of oral SDD for periodontitis in dogs, because the antimicrobial dose of doxycycline also inhibits MMPs. To treat chronic periodontitis, it would be necessary to identify the proper SDD for long-term use in dogs.

Several analytical methods exist for the determination of doxycycline in serum samples of various species of animals (Bennett *et al*., 1966; Kleibeuker *et al*., 2009;

Pijpers *et al*., 1991; Ruz *et al*., 2004; Wilson *et al*., 1988; Yu *et al*., 1992). High-performance liquid chromatography (HPLC) is rapid, simple and sensitive enough to determine serum doxycycline concentration with microsamples, and has been successfully used in previous experimental studies (Ruz *et al*., 2004; Yu *et al*., 1992). In addition, it would be appropriate to ascertain the MMP inhibitory effect in periodontal tissue to evaluate the efficacy of long- term SDD treatment in dogs. This study was performed to identify the optimal oral SDD for the treatment of periodontitis. The desired dose would not result in a serum doxycycline level that exceeded MIC, and would present clinical improvement with the MMPs inhibitory effect in periodontal inflammatory tissue after the SDD treatment in dogs.

Materials and Methods

The protocol of this study was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-100325-4).

1. Assessment of serum doxycycline levels

Twenty, approximately 1.5-year-old, clinically healthy Beagle dogs were used. Exclusion criterion for experimental animal selection was the administration of any systemic medications during the 2 weeks prior to the study. The dogs were equally and randomly divided into four groups and given doxycycline (Doxycycline hyclate, DongKoo Pharm. Co. Ltd., Seoul, Korea) respectively at a single dose of 1 (Group 1, n=5), 2 (Group 2, n=5), 3 (Group 3, n=5) and 5 (Group 4, n=5) mg/kg. Doxycycline was contained in the gelatin capsule and was administrated orally after the 6-hour fasting. Blood samples (1.7 ml) were collected from the jugular vein before the administration of doxycycline and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 hours post-administration. The blood was clotted in the serum-separating tube (SST^{TM} Tube, BD, Franklin Lakes, USA), and serum was obtained by centrifugation at 4000 rpm for 10 minutes.

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

Frozen samples and standard solutions were thawed for 60 minutes at 4°C and 200 µl of samples were mixed with the same volume of acetonitrile (Acetonitrile, HPLC grade, Mallinckrodt Baker Inc., Phillipsburg, USA). The mixtures were vortex-mixed for 1 minute, and centrifuged at 10000 rpm and 4°C for 10 minutes. After the centrifugation, the supernatants were diluted with the same volume of methanol (Methanol, Mallinckrodt Baker Inc., Phillipsburg, USA)-acetic acid (Acetid acid, Yakuri Pure Chemicals Co. Ltd., Osaka, Japan) mixture (1:1. v/v), and transferred to autosampler vials (Clear glass 12 x 32mm screw neck vial with cap and preslit PTFE/silicone septum, Waters Co., Milford, USA).

The HPLC system (Series 200 LC, PerkinEhlmer Inc., Shelton, USA) was coupled with an ultraviolet detector set at 347 nm, which was run using a software (TotalChrom Workstation version 6.3.1., PerkinElmer Inc., Shelton, USA). The technique used for HPLC analysis was a modified method of that previously described (Ruz *et al*., 2004). Serum samples were analyzed using a 150 × 4.6 mm, C_{18}, 5 µm silica reversed phase column (Alltima C18 5µ, Grace Davison Discovery Science, Deerfield, USA). Mobile phase was made by mixing 5% acetic acid, acetonitrile and methanol (55:25:20, v/v/v). Isocreatic solvent elution was performed at a flow rate of 1.0 ml/min.

2. Clinical and biochemical effects of SDD for periodontitis

Fifteen, 3-to-5-year-old Beagles with moderate-to-severe periodontitis were used to assess the efficacy of SDD. The dogs were confirmed to be generally healthy through a physical examination and had not received any systemic medication during 2 weeks prior to the study. All experimental operations and examinations were performed with dogs under general anesthesia induced by a combination of medetomidine (0.01 mg/kg; Domitor®, Orion Pharma, Espoo, Finland), tramadol (2 mg/kg; Toranzin inj., Samsung Pharm. Ind. Co., Ltd., Seoul, Korea), and commercial combination of zolazepam and tiletamine (2.5 mg/kg; Zoletil® 50, Virbac Laboratories, Carros, France) administered via intramuscular injection.

The examination sites were one to three premolars and molars for each dog selected on the basis of existence of clinical attachment loss (CAL; ≥ 1 mm/tooth) or bleeding on probing (BoP; ≥ 0.33/tooth). CAL was evaluated measuring the distance between the cemento-enamel junction and the bottom of probeable pocket (Wennström *et al*., 2001). BoP was scored 0 or 1 as the absent or the present of the bleeding within 10 seconds following probing (Wennström *et al*., 2001). The dogs were randomly divided into three groups according to the systemic administration dose of doxycycline. Group A (n=5; teeth=10), B (n=5; teeth=10), and C (n=5; teeth=10) received vehicle only, 1 mg/kg/day, and 2 mg/kg/day, respectively. All the medications were orally administrated 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 examined 4 weeks after the

administration of doxycycline, and the measurement was performed at the mesial-buccal, buccal and distal-buccal gingival margins per each tooth. All measurements were taken by one experienced clinician using a Williams periodontal probe (XP23-W Williams Explorer-Probe, Osung, Gimpo, Korea).

For the evaluation of the regional MMP inhibitory effect by the systemic administration of doxycycline, gelatin zymography was performed using full thickness gingival tissue. The sampling was performed on one to three separate sites from each dog assigned for the clinical evaluations. Before and 4 weeks after the daily doxycycline administration regimen, sample tissue was obtained from the buccal gingival margin. The approximately 1 x 2 mm-sized tissues were washed immediately using cold distilled water (4°C) to remove blood and debris and stored at -80°C until analyzed. Thawed gingival tissues were weighed and extracted at 4°C with lysis buffer (Tissue Extraction Reagent I, Invitrogen, Camarillo, USA) blended with protein cocktail (Protease Inhibitor Cocktail III, GenDEPOT, Barke, USA). The mixture ratio was 10 mg wet weight gingival tissue per 100 μl buffer. Protein in the gingival extracts was quantified by the Bradford method (Lovrien and Matulis, 2005). Twenty five micrograms of extracted tissue proteins were mixed with same volume of 2X zymography sample buffer (Novex® Tris-Glycine SDS Sample Buffer, Invitrogen, Carlsbad, USA) without heat denaturation. Electroporesis was performed using a 10% zymogram gel containing 0.1% gelatin at 125 V for 95 minutes. After electrophoresis, the gel was

incubated in 1X renaturating buffer (Novex® Zymogram Renaturating Buffer, Invitrogen, Carlsbad, USA) for 30 minutes at room temperature with gentle agitation and equilibrated in 1X developing buffer (Novex® Zymogram Developing Buffer, Invitrogen, Carlsbad, USA) in the same condition. The gel was incubated in fresh developing buffer at 37°C for 8 hours. Following the incubation, the gel was stained with 0.1% Coomassie Blue (Coomassie Brilliant Blue R250, Amresco, Solon, USA) and destained with 10% acetic acid in 40% methanol. Prestained protein marker (Xpert Prestained Protein Marker, GenDEPOT, Barke, USA) was run on each gel to identify the molecular size of gelatinase included in the samples. The intensities of the destained bands were determined by a software program (Multi-gauge V.3.0, Fujifilm, Tokyo, Japan) following the gel scanning process using a luminescent image analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

3. Statistical analyses

Statistical analyses were performed by use of a commercial software program (PASW statistics 18, SPSS Inc., Chicago, USA). Serum doxycycline concentration of each group was expressed as mean ± standard deviation (SD). To evaluate differences of the serum doxycycline concentration change within a treatment, the data were analyzed by repeated measures analysis of variance (repeated ANOVA). The correlation between orally administrated doxycycline and maximum doxycycline concentration in serum was calculated by the Pearson coefficient for correlation.

To evaluate the clinical effect of SDD, the clinical parameters of week 0 and week 4 in same group were assessed using a paired t-test to compare periodontal status on the same tooth within each group. One-way analysis of variance (ANOVA) was used for the intergroup comparison of the clinical periodontal parameters on weeks 0 and 4, and the variance of gelatinolytic activity between week 0 and week 4. The baseline gelatinolytic values (week 0) were also compared using one-way ANOVA to evaluate the statistical difference among the three medication groups. Tukey method was used as a *post hoc* test and the data within 95% confidence level were considered statistically significant.

Results

1. Doxycycline concentrations in the serum

Changes in the mean serum doxycycline concentration before and after administration were presented (Fig. 6). The mean HPLC retention time for doxycycline was 2.94 ± 0.19 minutes. After initial administration, serum doxycycline reached a maximum concentration at 3 to 4 hours, and the serum doxycycline levels were maintained below the MIC for 24 hours in Groups 1 and 2 (Fig. 6). The mean serum doxycycline concentration of each group showed significant difference according to the flow time ($P<0.05$). The correlation between the amount of administration and detectable concentration in serum was statistically significant ($r=0.717$; $P<0.05$).

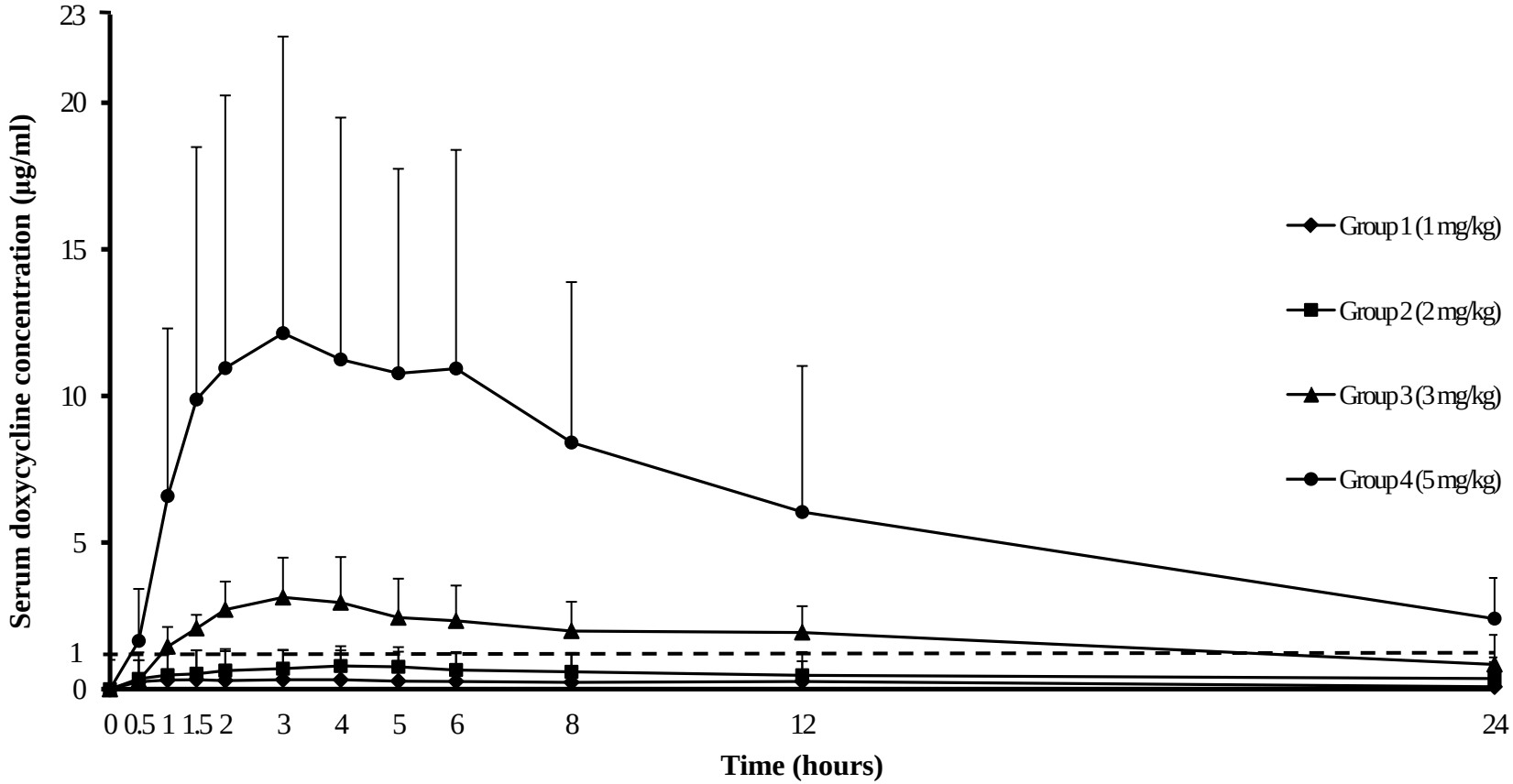


Fig. 6. Change in mean serum doxycycline concentrations after an oral administration of doxycycline in beagles.

2. Clinical effects of SDD for periodontitis

The baselines of clinical parameters including CAL and BoP were not significantly different on week 0. Mean ± SD of CAL and BoP on weeks 0 and 4 were presented and compared (Table 3). After the 4-week medication period, Group C significantly decreased in CAL compared to the value at week 0 and showed significant lower CAL than the other groups ($P<0.05$). On week 4, the BoP score of Group C was significantly decreased, whereas the other groups were increased ($P<0.05$). However, the variations compared to the value at week 0 were not significant.

Table 3. Clinical periodontal parameters

Week	Parameter	Group A	Group B	Group C
Week 0	CAL	2.67 ± 1.45	2.67 ± 1.35	2.23 ± 1.33
	BoP	0.8 ± 0.4	0.8 ± 0.4	0.7 ± 0.5
Week 4	CAL	2.53 ± 1.11[a]	2.53 ± 1.25[a]	1.83 ± 0.83[b,*]
	BoP	0.9 ± 0.3[a]	0.9 ± 0.3[a]	0.6 ± 0.5[b]

Data are expressed as the mean ± SD (n=5; 10 teeth). [a,b]Across each row, values with different superscripts are significantly different. [*]Significantly decreased value compared to the same parameters of week 0 in same group. Group A: placebo, once a day, Group B: 1 mg/kg of doxycycline once a day, Group C: 2 mg/kg of doxycycline once a day. CAL: Clinical attachment loss, BoP: Bleeding on probing.

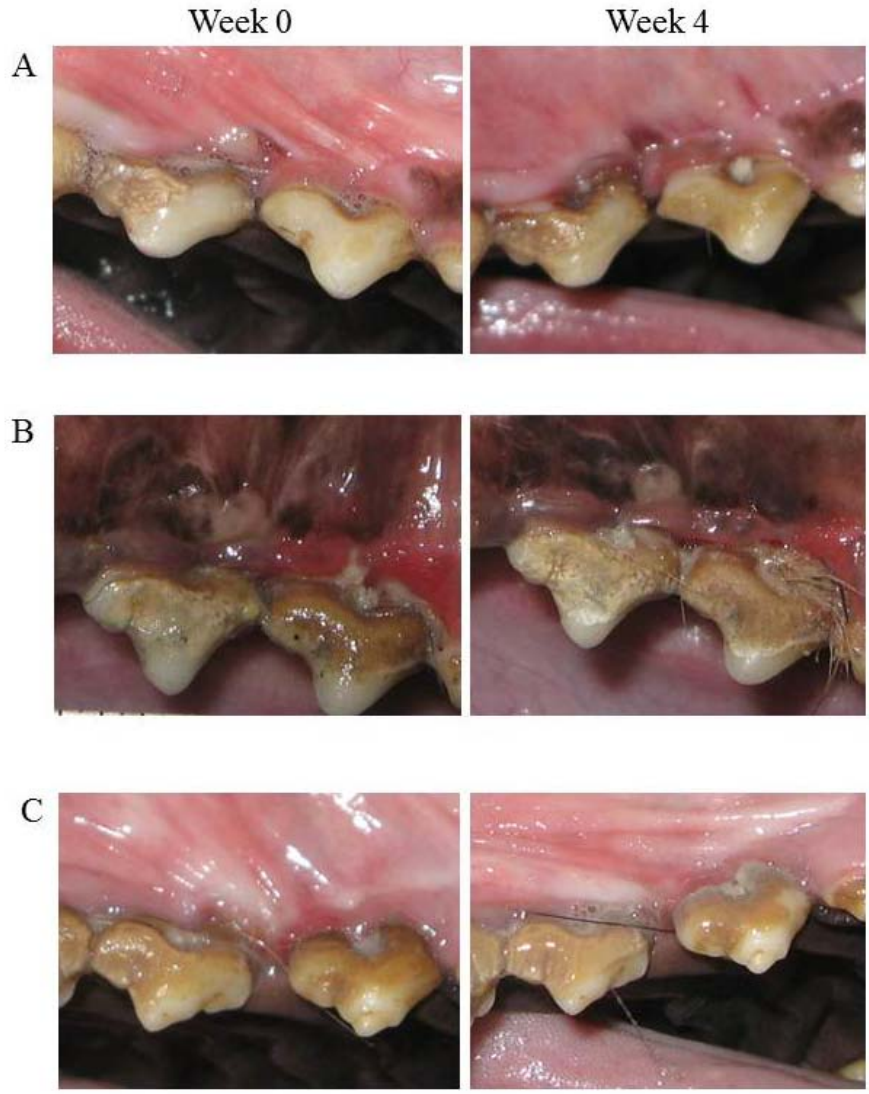


Fig. 7. Representative clinical periodontal status before and after 4-week medication. A. Group A (placebo, once a day), B. Group B (1 mg/kg of doxycycline, once a day), C. Group C (2 mg/kg of doxycycline, once a day).

3. Biochemical effects of SDD for periodontitis

In the zymographic evaluation, gelatinolytic bands were evident in all gingival samples at the 92 kDa and below 72 kDa areas (Fig. 8). Mean ± SD of baseline gelatinolytic intensities at the 92 kDa and below 72 kDa areas, and the variations between 0 to 4 weeks according to the groups were summarized (Table 4). The baseline intensities were not significantly different among the groups. However, the zymographic intensities on the both molecular weights were increased in Group A, while the other groups displayed decreased intensity after the 4-week medications. The variation was significantly different in Group A compared to the other groups ($P<0.05$).

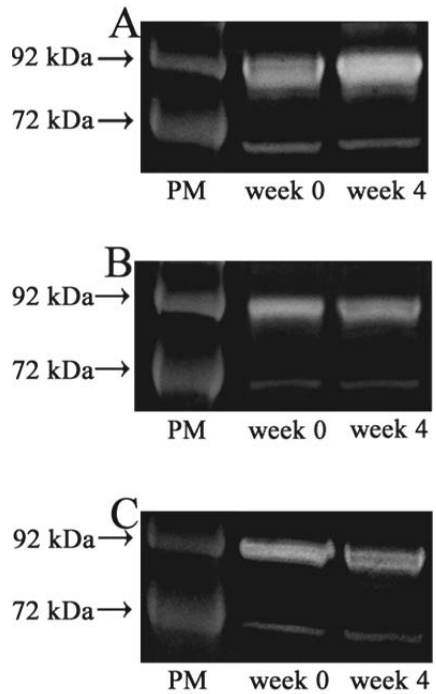


Fig. 8. Representative gelatinolytic intensities comparing week 0 and week 4 in the same periodontal sampling sites. A. Group A (placebo, once a day), B. Group B (1 mg/kg of doxycycline, once a day), C. Group C (2 mg/kg of doxycycline, once a day). PM: Prestained protein markers (Xpert Prestained Protein Marker, GenDEPOT, Barke, USA)

Table 4. Gelatinolytic intensities and the relative variations for 4 weeks

MMPs (Molecular Weight)	Groups	Week 0 (AU)	Week 4 (AU)	Variation of zymographic intensity (-fold changes)
MMP-9 (92 kDa)	Group A	162908.02 ± 96988.48	198439.90 ± 119743.49*	1.29 ± 0.35*
	Group B	105017.01 ± 89518.88	88782.09 ± 86515.17†	0.82 ± 0.27†
	Group C	150371.73 ± 60799.43	132924.08 ± 61878.73*,†	0.85 ± 0.16†
MMP-2 (below 72 kDa)	Group A	96676.43 ± 35103.62	124537.10 ± 66853.04^{a}	1.31 ± 0.55^{a}
	Group B	59774.34 ± 34235.34	41648.80 ± 32858.28^{b}	0.65 ± 0.34^{b}
	Group C	67280.69 ± 35243.54	42457.11 ± 56361.93^{b}	0.66 ± 0.58^{b}

*,†Across each column in MMP-9 section, values with different superscripts are significantly different. ^{a,b}Across each column in the MMP-2 section, values with different superscripts are significantly different. Group A: placebo, once a day, Group B: 1 mg/kg of doxycycline, once a day, Group C: 2 mg/kg of doxycycline, once a day. AU: Arbitrary unit in the densitometer (Multi-gauge V.3.0, Fujifilm, Tokyo, Japan).

Discussion

Tetracyclines are generally used as bacteriostatic antibiotics. However, tetracycline analogues additionally possess non-antibiotic properties, namely inhibition of MMPs. Because of these host-modulatory effects, doxycycline has been used for the treatment of inflammatory diseases such as osteoarthritis and refractory corneal ulcer in dogs (Chandler *et al*., 2010; TeKoppele *et al*., 1998; Yu *et al*., 1996). Furthermore, the effects of the topical application of doxycycline for the treatment of periodontitis have been investigated, and a commercial preparation is available for use in dogs (Polson *et al*., 1996; Zetner and Rothmueller, 2002). However, the method might be a one-time remedy administered under general anesthesia in dogs, with the effects lasting only several days or weeks (Golub *et al*., 1991; Polson *et al*., 1996; Zetner and Rothmueller, 2002). Furthermore, antimicrobial susceptibility changes could occur with a long-term systemic administration of antimicrobial-doses of doxycycline.

Previous studies demonstrated the benefit of SDD to periodontal disease as a systemic host-modulatory therapy in human patients (Caton and Ryan, 2011). SDD of humans has been established as 20 mg/person twice a day; this dose does not exert a subgingival antimicrobial effect and does not lead to changes in antimicrobial susceptibility, even during long-term use (Thomas *et al*., 2000; Walker *et al*., 2000). The SDD is about one-fifth of the general antimicrobial dose and was experimentally confirmed to yield serum concentrations of 0.6 to 0.8 μg/ml, which are considerably below the MIC of subgingival microflora, determined *in vitro* (Caton, 1999; Walker *et al*., 2000). Additionally, the genera of canine subgingival pathogenic anaerobes are similar to that of humans (e.g.,

Porphyromonas, *Bacteroides*, and *Prevotella*), although the specific species were different (Dahlén 1993; Radice *et al*., 2006; Hardham *et al*., 2005). Considering these aspects, the present results revealed that the mean serum doxycycline concentration of Groups 1 and 2 was maintained considerably below 1 μg/ml, which was the MIC of subgingival microflora, during the experimental phase.

Research concerning the pharmacokinetics of doxycycline polyphosphate in dogs had revealed that the maximum concentration-time and the half-life of orally administrated doxycycline were 2.8 and 11.8 hours, respectively (Michel *et al*., 1979). The maximum concentration-time was similar that of the present study, however the half-life tended to be extended to over 12 hours in all groups. Therefore, the serum doxycycline concentration could exceed the MIC by the periodic twice-a-day administration even in Group 2. In addition, it was estimated that the 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 maintain below the MIC for the clinical use of SDD in dogs, even though long-term medication might be continued. A recent study reported that long-term oral administration of 40 mg of doxycycline once a day produced no antimicrobial resistance, which was also effective for the improvement of periodontitis in human (Preshaw *et al*., 2008). This regimen was designed as a novel, sustained-release formulation to produce subantimicrobial blood levels of doxycycline for the patient compliance, because it is well-known that once-daily dosing is preferred compared to more frequent dosing due to the compliance, especially in long-term medication (Preshaw *et al*., 2008). In pharmacokinetic aspects, 1 mg/kg, twice-daily dosing or 2 mg/kg, once-daily

dosing could be suggested to avoid development of antimicrobial resistance in dogs in the present study. However, daily administration of 2 mg/kg doxycycline would be more suitable for the consideration of patient compliance.

There were some limitations in the present study involving the poor detection of doxycycline in some blood samples of the lowest dose group (Group 1). Also, the number of dogs in each group was small, hindering definitive conclusions. A previous study revealed that the confirmed concentration range using this HPLC method was 0.4-80 μg/ml for doxycycline quantification in serum (Ruz *et al*., 2004). In the present study, the average of the maximum serum-doxycycline concentration of Group 1 did not attain 0.4 μg/ml. Thus, the serum doxycycline concentration of Group 1 might be underestimated slightly. However, the amount of administered doxycycline significantly correlated with the serum concentration of doxycycline by Pearson coefficient for correlation. The data from the present study prove that the serum doxycycline concentrations differ significantly according to the administrated dose and time flow by repeated measures analysis of variance. It can be concluded that the doxycycline concentrations of all groups were reliable and whose average was within the concentration range.

The 92 kDa form of gelatinase is the most prominent form in the gingival tissues of human patients with chronic periodontitis (Golub *et al*., 1995; Lee *et al*., 2004). In another study, MMP-2 expression in gingival tissue was correlated with the periodontal pathologic status (Dong *et al*., 2009). The 72 kDa and 92 kDa species represent MMP-2 and -9, respectively, and could be detected from diseased periodontium using gelatin zymography (Golub *et al*., 1995; Lee *et al*., 2004; Snoek-van Beurden and Von den Hoff, 2005). Therefore, the present study used

molecular weight standards to determine the type of the gelatinase. In the present zymographic results, a single gelatinolytic band was located below 72 kDa, which could be regarded as the active-form of MMP-2. In contrast, the bands located around 92kDa comprised double layers, which were considered as a mix of pro-MMP-9 and activated-MMP-9 on week 0.

Although the baseline averages were not exactly the same, gelatinolytic activity was not significantly different in the present study. Therefore, this study used the method involving variation of the gelatinolytic activity in the same sampling site. According to the zymographic results, both MMP-2 and -9 were significantly decreased in Groups B and C, while Group A displayed increased-expression of the MMPs after the one-month administration of SDD. Previous studies revealed that polymorphonuclear leukocyte-derived MMPs can be directly inhibited by SDD in humans (Choi *et al*., 2004; Golub *et al*., 1990). Furthermore, the active-form MMPs can be directly inhibited in part by doxycycline (Golub *et al*., 1995). In the present study, both pro- and active-MMP-9 were increased in Group A, while Groups B and C showed 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 reduces the expression of pro-MMP-9, but also might inhibit the conversion of the pro-form into the activated-form in dogs. Although the present zymography result showed no statistically significant difference, the clinical parameters of Group C were significantly different compared to the other groups after the medication period. Considering these results, 2 mg/kg in the once a day regimen could be used for the clinical improvement of diseased periodontal status which showed MMP-2 and -9 inhibition without antimicrobial effect.

Conclusions

Considering the HPLC, clinical and zymographic results, it is suggested that 2 mg/kg doxycycline administration once a day would be an appropriate dose for the subantimicrobial dose in dogs. According to these results, the subantimicrobial dose could be recommended for long-term treatment of gelatinolytic inflammatory diseases such as periodontitis and arthritis. Because the removal of injurious bacteria should be performed prior to the other treatment for the periodontitis, the medication protocol should be established which combined with subgingival scaling before SDD treatment. Before clinical application of SDD in dogs, further *in vivo* studies including the other biomarkers such as the other MMPs activity, tissue inhibitor of metalloproteinase-1 and interleukin-6 levels, which support clinical improvements and antimicrobial resistance, would be needed for long-term use.

CHAPTER III.

Clinical and Microbiological Effects of Subantimicrobial Dose of Oral Doxycycline on Periodontitis in Dogs

Abstract

This study was designed to demonstrate the influence of the oral administration of a subantimicrobial dose of doxycycline (SDD) for normal periodontal flora and antimicrobial susceptibility. Experimental periodontitis was induced on five premolars and one molar of 12 healthy beagles using a silk and wire-twisted ligature for 60 days. After each periodontitis induction period, the ligature was removed, and subgingival scaling was performed. The dogs were randomly assigned to one of two groups and medicated for 8 weeks: 2 mg/kg of doxycycline (SDD group) or a placebo capsule (control group) once a day. On weeks 0, 4 and 8, the clinical parameters including plaque index, gingival index, periodontal pocket depth, clinical attachment level and bleeding on probing were evaluated. After the clinical assessments, subgingival plaque was sampled and then cultured in an anaerobic system for one week and the total anaerobes, *Porphyromonas* spp, *Bacteroid* spp and *Pasturella* spp counts were investigated. Using the agar dilution method, the minimum bactericidal concentration of doxycycline was evaluated and the resistance for doxycycline was monitored during this experimental phase. The clinical periodontal status of the SDD group was significantly improved compared to the control group. All bacterial counts were not significantly different between the two experimental groups. Antibacterial resistance was not established in the SDD group during the experimental periods ($P<0.05$). These results suggest that the once a day oral regimen of 2 mg/kg of doxycycline could serve as a SDD in dogs.

Introduction

Doxycycline is a member of tetracyclines that is effective in the treatment of periodontal disease (Golub *et al*., 1991). Doxycycline has an antimicrobial effect and also an anti-inflammatory effect due to inhibition of matrix metalloproteinases (MMPs) (Choi *et al*., 2004; Emingil *et al*., 2004a; Golub *et al*., 1991; 1995; Lee *et al*., 2004). Long-term administration of antibiotics could lead to antimicrobial resistance, and the doxcycline-mediated inhibition of MMPs occurs below the antimicrobial dose (Lee *et al*., 2004). Therefore, the approach involving a subantimicrobial dose of doxycycline (SDD) has been applied to the treatment of inflammatory lesions, including periodontitis and arthritis, which occur due to the host response in humans (Nordström *et al*., 1998). Previous studies demonstrated that the administration of SDD with subgingival root planning can resolve chronic periodontal inflammation (Choi *et al*., 2004; Emingil *et al*., 2004a; 2004b; Golub *et al*., 1995). Furthermore, the long-term administration of SDD did not induce antimicrobial effects on the subgingival microflora and did not affect the antimicrobial susceptibility in humans (Thomas *et al*., 2000; Walker *et al*., 2000).

In contrast, the effect of SDD in dogs has not been determined for routine therapeutic use, including periodontal treatment. In chapter II, SDD in dogs was evaluated using high-performance liquid chromatography, and the efficacy was revealed in middle-aged dogs with periodontitis. However, the SDD efficacy study in chapter II used uncontrolled periodontitis-predisposing animals and did not consider the environmental variables, including calculus and the aspect of periodontal inflammation, which could affect the prognosis. Furthermore, the alteration of subgingival microflora and the antibiotic resistance after the

administration of SDD was not evaluated. The periodontitis could worsen, due to the overgrowth of more resistant and pathogenic microorganisms, if the accumulation of SDD suppresses the normal susceptible microflora (Edlund *et al*., 1996).

In human medicine, it has been shown that SDD treatment as an adjunct to scaling was effective and did not affect the subgingival microflora (Caton and Ryan, 2011). This study was performed to evaluate the effect of SDD as an adjunctive therapy to subgingival scaling, and to assess the alteration of subgingival microflora and their antimicrobial resistance after the 2-month administration.

Materials and Methods

The protocol of this study was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-100609-4). Twelve, approximately 1.5-year-old conventional beagles (eight females and four males) without periodontal disease, were used in this study. Exclusion criterion for experimental animal selection was the administration of any systemic medications during the 1-month prior to the study. All experimental procedures including sampling and clinical periodontal evaluations were performed under general anesthesia with a combination of medetomidine (0.01 mg/kg; Domitor®, Orion Pharma, Espoo, Finland), tramadol (2 mg/kg; Toranzin inj., Samsung Pharm., Seoul, Korea) and commercial combination of zolazepam and tiletamine (2.5 mg/kg; Zoletil® 50, Virbac Laboratories, Carros, France) by an intramuscular injection.

1. Experimentally induced periodontitis

For the preparation of healthy gingiva, all teeth were scaled and polished using a piezoelectric ultrasonic scaler (ART-SP2, Bonart Co., Ltd, La Puente, USA), and tooth brushing was performed once a day without anesthesia for the following 2 weeks. During these prophylactic periods, the beagles were fed a hard pellet diet to reduce plaque formation (Martuscelli *et al*., 2000). After the preparation period, experimental periodontitis was induced on the left maxillary 2nd premolar (PM2), 3rd premolar (PM3) and 4th premolar (PM4), as well as the left mandibular PM3, PM4 and 1st molar (M1) using a twisted-wire (Spooled ligature wire; ClassOne Orthodontics, Lubbock, USA) with 2-0 silk (Silk 2-0, Ailee Co., Ltd., Busan, Korea) ligature as described in chapter I. For the promotion of plaque formation, soft-moistened food was given for the following 60 days. The ligatures were checked daily and the lost ligatures were repaired immediately.

2. Evaluation of clinical effects of SDD on periodontitis

Sixty days after the periodontitis induction, the ligatures were removed and the clinical periodontal parameters were recorded prior to the initiation of treatment to evaluate the baseline (week 0) periodontal status. The clinical parameters included the plaque index (PI), gingival index (GI), periodontal pocket depth (PPD), clinical attachment loss (CAL) and bleeding on probing (BoP) (Löe and Silness, 1963; Silness and Löe, 1964; Wennström *et al*., 2001). The measurements were performed at the mesial-buccal, buccal and distal-buccal gingival margins of each tooth. Subgingival plaque samples were taken from the left maxillary PM4 and left mandibular M1. Collected samples were weighed and stored below 4ºC in coded micro-centrifuge tubes until they were cultured. After the clinical examination and sampling, all dogs received sub-and supra-gingival ultrasonic scaling on week 0. The dogs were randomly divided into two groups depending on whether a subantimicrobial dose of oral doxycycline would be administrated or not. The SDD group (n=6, 36 teeth) and control group (Placebo; n=6, 36 teeth), received 2 mg/kg/day of doxycycline which were contained in the gelatin capsules and vehicles only, respectively. All the medications were orally administrated for 8 weeks, 30 minutes after every morning meal, once a day. The clinical conditions of each dog were checked daily. On weeks 4 and 8, their clinical periodontal status was reevaluated and subgingival plaque samples were recollected in the same manner. All measurements and collection of samples were taken by a single experienced clinician using a Williams periodontal probe (XP23-W

Williams Explorer-Probe, Osung, Gimpo, Korea) and a curette (GR3-4 Hu-friedy type Gracey curette, Osung, Gimpo, Korea), respectively.

3. Periodontal plaque culture

The sampled plaque and calculus were diluted 100-fold into anaerobic-sterilized and lactated Ringers solutions. The diluted solutions were gently sonicated to scatter the plaque and were re-diluted 10^4-fold. After vortexing, 100 μl aliquots of 10^{-6} solutions were dispensed onto commercial Trypticase-soy blood agar (TSBA) plates containing 5% sheep blood (TSBA plates, Hankang Media Co., Gunpo, Korea) and spread with sterile glass rods. The plates were incubated in a completely anaerobic system (AnaeroPack™-Anaero; Rectangular jar, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C for 1 week. After the incubation, each sample was assessed by counting visible colonies, which were separately counted according to the morphology of each colony with or without hemolysis. Total anaerobic counts were also evaluated. The sampled colonies were sent to a laboratory (Macrogen Inc., Seoul, Korea), and the colonies were identified by 16s rRNA sequencing using universal primer. Finally, the counts of the three most predominant species were evaluated.

The minimal bactericidal concentration (MBC) of doxycycline was also determined on week 0. The MBC of doxycycline was determined for the plaque suspension by the modified agar dilution technique in a range of concentrations between 1-16 μg/ml using a TSBA plate (Walker *et al*., 1979). The plates were also incubated in a completely anaerobic environment at 37°C for 1 week. The lowest doxycycline concentration that yielded no growth of a visible colony was considered as the MBC. To evaluate the alteration of antimicrobial resistance, the plaque dilutions

sampled at week 8 were cultured again on the doxycycline diluted TSBA plate, which contained the titrated MBC of doxycycline at week 0.

4. Statistical analyses

Clinical periodontal parameters of each group were expressed as mean ± standard deviation (SD). Statistical analyses were performed by use of a commercial software program (PASW 18.0, SPSS Inc., Chicago, USA). To evaluate the differences of clinical effect of SDD and subgingival bacterial counts according to the weeks, the values of weeks 0, 4 and 8 were assessed using a repeated measures analysis of variance (repeated ANOVA). The Tukey method was used as a *post hoc* test. To compare periodontal status and the bacterial counts between the groups, the values of each week were also compared using a Student's *t*-test. The data within 95% confidence level were considered statistically significant.

Results

1. Periodontal status

Mean ± SD of periodontal indices on weeks 0, 4 and 8 were presented (Table 5). The baseline values of all periodontal parameters were not significantly different between the two groups on week 0. Four weeks after the medication, both groups showed significant within-treatment differences relative to week 0 ($P<0.05$). The periodontal status of week 8 was not significantly different compared to week 4, but was compared to week 0 ($P<0.05$). In intergroup comparison, all of the periodontal status parameters (PI, GI, PPD, CAL and BoP) were significantly improved in the SDD group compared to the control group on weeks 4 and 8 ($P<0.05$).

Table 5. Clinical periodontal parameters

Group	Parameters	Week 0	Week 4	Week 8
Control	PI	2.6 ± 0.7	2.2 ± 0.8*	2.2 ± 0.7*
	GI	2.3 ± 0.5	1.9 ± 0.5*	2.0 ± 0.5*
	PPD	1.92 ±0.88	1.74 ± 0.62*	1.77 ± 0.61*
	CAL	1.84 ± 0.85	1.61 ± 0.93*	1.52 ± 0.91*
	BoP	0.7 ± 0.5	0.7 ± 0.5*	0.6 ± 0.5*
SDD	PI	2.7 ± 0.5	1.9 ± 0.7*,†	1.9 ± 0.8*,†
	GI	2.2 ± 0.5	1.5 ± 0.5*,†	1.5 ± 0.7*,†
	PPD	1.80 ± 0.70	1.45 ± 0.53*,†	1.34 ± 0.57*,†
	CAL	1.63 ± 0.81	0.99 ± 0.75*,†	1.18 ± 0.88*,†
	BoP	0.7 ± 0.5	0.3 ± 0.5*,†	0.3 ± 0.7*,†

*Statistically significant within-treatment differences relative to week 0. †Statistically significant differences between control and SDD groups at the same week. Control: placebo, once a day, SDD: 2 mg/kg of doxycycline, once a day. PI: plaque index, GI: gingival index, PPD: periodontal pocket depth, CAL: clinical attachment level, BoP: bleeding on probing.

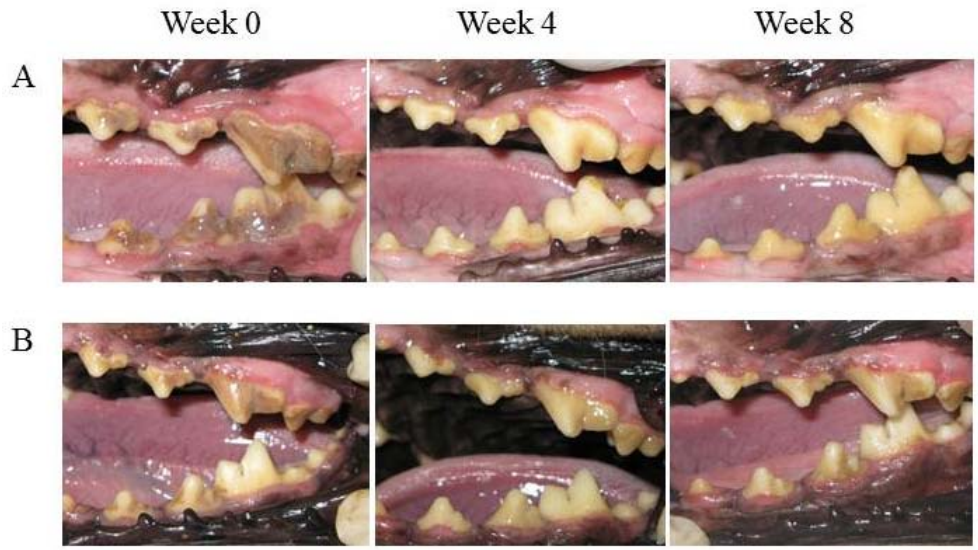


Fig. 9. Representative clinical periodontal status before and after mediation. A. Control group (placebo, once a day), B. SDD group (2 mg/kg of doxycycline, once a day).

2. Subgingival microflora and antimicrobial resistance

For the periodontal microbiological assessment, six subgingival samples per group were collected. Twenty morphologically different colonies were isolated and the 16s rRNA sequencing results revealed that nine different species of anaerobes were present (Table 6). The most predominant species in the subgingival flora, in order of prevalence, were *Porphyromonas* spp., *Bacteroid* spp. and *Pasturella* spp. The variation of anaerobic bacterial counts were compared between the groups (Fig. 10). The number of total anaerobic bacteria, *Porphyromonas* spp., *Bacteroid* spp. and *Pasturella* spp. isolates were not significantly different between the two treatments on weeks 0, 4 and 8. Within the same group, no significant differences were found during the course of experimental periods.

The evaluated MBC of doxycycline for the total anaerobes was 16 μg/ml on week 0. On week 8, a few colonies were isolated from the agar plate which contained 16 μg/ml of doxycycline in both groups. The number of dogs with bacterial growth on the MBC of the doxycycline-deluted agar plates was presented (Table 7). And it was also presented that the mean ± SD of the colonies including *Porphyromonas* spp., *Bacteroid* spp. and *Pasturella* spp (Table 7). In the intergroup comparison, the bacterial growths of the three species were not significantly different between control and SDD groups.

Table 6. Percentages of subgingival anaerobes identified by 16s rRNA sequencing before and after the 8-week administration of SDD

Species	No. of subspecies	Control (%)		SDD (%)	
		Week 0	Week 8	Week 0	Week 8
Bacteroides spp.	3	20.74	32.06	24.21	24.75
Campylobacter spp.	1	4.28	0	7.55	0
Desulfovibrio spp.	1	9.13	0	12.11	0
Fusobacterium spp.	1	0	0.48	0.11	0
Haemophilus spp.	1	1.00	3.62	2.76	5.28
Pasturella spp.	2	13.46	14.19	17.96	15.85
Porphyromonas spp.	2	49.81	43.25	34.68	50.70
Propionibacterium spp.	1	0.33	0	0.28	0
Streptococcus spp.	1	1.24	6.41	0.34	3.41

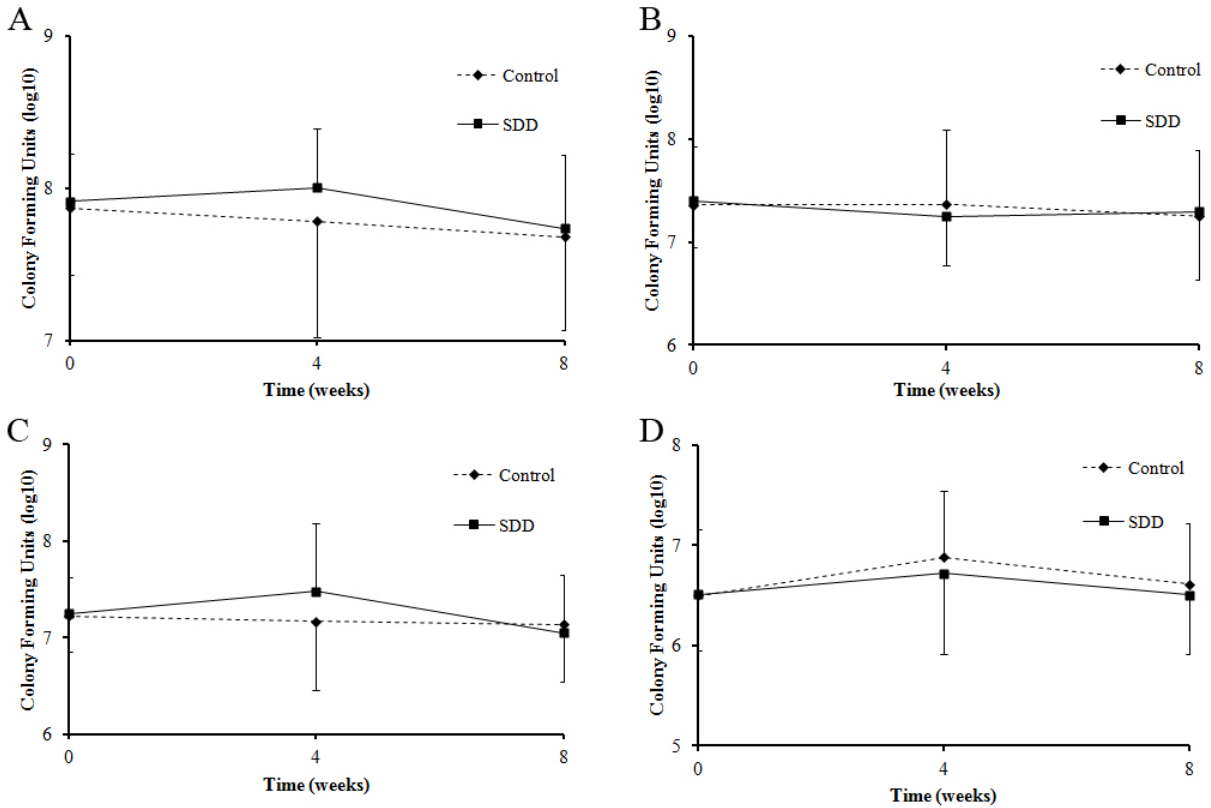


Fig. 10. The variation of cultivable anaerobic microbial flora during experimental periods. A. Total anaerobic counts, B. Total *Porphyromonas* spp. counts, C. Total *Bacteroides* spp. counts, D. Total *Pasturella* spp. counts.

Table 7. The number of dogs with bacterial growth on the MBC of doxycycline-diluted agar plate and the number of colonies per plate

	Number of dogs		Colonies per plate		P value
	Control (N=6)	SDD (N=6)	Control (n=18)	SDD (n=18)	
Porphyromonas spp.	2	2	0.1 ± 0.3	0.4 ± 0.9	0.20
Bacteroides spp.	3	2	1.3 ± 2.0	0.7 ± 1.7	0.38
Pasturella spp.	1	1	0.1 ± 0.2	0.3 ± 1.0	0.25

MBC: minimal bactericidal concentration. Control group: placebo, once a day, SDD group: 2 mg/kg of doxycycline, once a day. N: number of dogs, n: number of plates.

Discussion

This study was designed to identify whether the SDD would not affect subgingival microflora while improving the periodontal status. The SDD was evaluated employing the biochemical method in chapter II. In the present study, the clinical periodontal parameters were significantly improved between weeks 0 and 4, but there were no significant changes between weeks 4 and 8 in both groups. These observations suggested that the significant improvement of periodontal status in the control group was due to the periodontal debridement using an ultrasonic scaler. However, the SDD group showed significantly more improvement of periodontal status compared to the control group. Considering these aspects, the proper SDD administration period to improve clinical status could be approximately one month.

In canine patients, the periodontal application of doxycycline has been used as an adjunct to scaling for the treatment of periodontitis (Zetner and Rothmueller, 2002). However, local administration of doxycycline should be performed under general anesthesia in dogs. Furthermore, the systemic long-term administration of an antimicrobial dose of doxycycline could lead to the antibiotic resistance of normal subgingival microflora. For these reasons, a study was undertaken (see chapter II) to assess the systemic dose of doxycycline that would not exceed the minimal inhibitory concentration of serum doxycycline. In this study, the dose evaluated from chapter II (2 mg/kg, once a day) was used as an adjunctive therapy to dental scaling. The SDD did not evoke an antimicrobial effect after the 8-week administration. The antimicrobial effect was evaluated using an anaerobic culture of subgingival microorganisms on the MBC of doxycycline-diluted TSBA.

The minimal inhibitory concentration (MIC) was commonly measured, one for each microbial species, so subculture of the individual colony should be performed. However, the distribution and predominance of bacterial species were different in all dogs (Dahlén *et al*., 2012). In general, a known subgingival microflora included more than 20 species in dogs and the plaque formation could be accomplished by the interaction of different species of subgingival bacteria (Daep *et al*., 2008; Dahlén *et al*., 2012; Liang *et al*., 2011). Therefore, this study evaluated the MBC of predominant microflora on week 0 to compare that of week 8.

In the present study, the species were classified according to their morphologic features, and identified by 16s rRNA sequencing. Previous studies revealed that black-pigmented anaerobic bacteria from dog's subgingival flora were acted as the primary periopathogen, and the most frequently presented species was *Porphyromonas* spp (Dahlén *et al*., 2012; Hardham *et al*., 2005). Another study reported *Porphyromonas* spp. was present significantly more in the subgingival samples with periodontitis compared to the samples without (Senhorinho *et al*., 2011). A recent study described that the predominant species of the subgingival plaque included *Porphyromonas, Fusobacterium, Filifactor, Bacteroides, Peptostreptococcus, Pasturella, Campylobacter* and *Tannerella* (Dahlén *et al*., 2012). According to the results of the study, *Streptococcus* and *Actinomyces*, which are frequently found in humans, were not the predominant species in dogs, while *Pasturella* spp. was predominant (Dahlén *et al*., 2012). The results of a previous study were similar to the results of the present study. In the present study, the three most prevalent cultivable oral microbes were *Porphyromonas* spp., *Bacteroides* spp. and *Pasturella* spp. Considering these aspects, this study monitored not only the

total anaerobic bacterial counts, but also the *Porphyromonas*, *Bacteroides* and *Pasturella* spp counts. Consequently, the total counts of anaerobes according to the species were not significantly different, but a few colonies were grown on the MBC of doxycycline-diluted agar plate after the 8-week administration of SDD. However, the bacterial growth was not significantly different between the control and SDD groups, and the total counts were suppressed more than 100 times compared to the non-additive TSBA with the same concentration of plaque solution. These results suggested that the bacterial growth after the experimental phase was not due to outgrowth of SDD administration.

The ideal SDD of humans has been established as 20 mg per person, twice a day (Thomas *et al*., 2000; Walker *et al*., 2000). It was identified that no alteration of antimicrobial susceptibility and subgingival microflora occurred after 12-months of administrating period (Thomas *et al*., 2000; Walker *et al*., 2000). A more recent study revealed that 40 mg of doxycycline, with once daily dosing designed as a sustained-release formulation, produced no antimicrobial resistance in humans (Preshaw *et al*., 2008). Although the delayed-releasing capsule was not employed, the oral administration of 2 mg/kg once daily did not induce antimicrobial resistance and the periodontal status was significantly improved. This result reflected the result of chapter II that the half-life of SDD tended to be extended to over 12 hours. In addition, the 1 mg/kg twice daily regimen is also effective to the periodontal inflammatory lesion, as shown in chapter II. However, once-daily dosing is preferred compared to more frequent dosing to improve the compliance of the patient and client, especially in long-term medication.

Conclusions

In conclusion, the oral administration of 2 mg/kg of doxycycline once a day for 2 months was clinically effective for the treatment of periodontitis without alteration of the distribution of subgingival anaerobes and the antimicrobial susceptibility in dogs. These results suggest that the oral regimen of 2 mg/kg doxycycline once a day could serve as a SDD in dogs.

GENERAL CONCLUSIONS

This study was performed to evaluate the SDD which did not affect subgingival anaerobic bacteria and antimicrobial resistance but sustain the inhibitory effect of MMPs using standardized periodontitis model in dogs.

In chapter I, experimental periodontitis was induced using various kinds of ligatures, including silk, wire and twisted-ligature with silk and wire. The maintaining periods of each group were recorded and the variations of periodontal status during the experimental periods were evaluated. Among the experimental groups, twisted-ligature with silk and wire was maintained for a significantly long time compared to the silk ligature. Furthermore, the twisted ligature induced significantly advanced experimental periodontitis compared to the other groups by single induction during the same period. According to these results, the experimental periodontitis induced by the twisted-ligature with silk and wire could be an effective method for the investigation of periodontitis in dogs.

In chapter II, the diurnal variations of serum concentration of doxycycline were measured using HPLC after the oral administration of various doses of doxycycline. Mean serum doxycycline concentrations of 1 and 2 mg/kg groups were maintained significantly lower than the generally known minimal inhibitory concentration of doxycycline during 24 hours. Based on these results, 1 and 2 mg/kg once daily regimens were regarded as SDD. The MMPs expressions of the periodontium were measured using gelatin zymography before and after the one month administrations of SDD. The zymographic intensities which reflected MMPs expression were significantly decreased in the SDD administrated groups compared to placebo

group. In addition, clinical improvements of periodontal status were dose-dependent.

In chapter III, the subgingival plaque samples were cultured in the anaerobic system before and after the 2-month administration of SDD, which was determined in chapter II. The clinical parameters were recorded and the number of total anaerobes, *Porphyromonas* spp., *Bacteroid* spp. and *Pasturella* spp. were counted. In addition, the viabilities of anaerobes spreading on the doxycycline-diluted agar plates were monitored to evaluate the alterations of antimicrobial resistance. The clinical periodontal status of SDD group was significantly improved compared to the Placebo group. All kinds of bacterial counts were not significantly different between the two experimental groups. Antibacterial resistance was not established in the SDD group during the experimental periods.

Through these studies, it is demonstrated that per oral administration of 2 mg/kg doxycycline once a day would be significantly effective for the management of periodontitis in dogs. Therefore, this dose of doxycycline would be suitable for the SDD in dogs.

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국 문 초 록

개에서 치주염 치료를 위한 항생농도 이하 doxycycline의 경구투여에 대한 평가

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치주질환은 사람과 개에서 가장 호발하는 질병 중 하나이다. 만성적인 치주염의 치료를 위해서는 다른 처치보다 우선적으로 물리적인 치석 및 치태의 제거가 이루어져야 한다. 인의에서는 이러한 물리적 치주치료 이후에 지속적으로 치주염을 관리할 수 있는 약제들에 대한 연구가 많이 이루어져 있다. 이 중 항생농도 이하 doxycycline (SDD)의 사용은

치주염을 유발하는 주요 효소 중 하나인 matrix metalloproteinases (MMPs)의 활성을 저하시키는 방법으로, 치석 제거 이후에 보조적으로 사용할 수 있는 치료법 중 하나이다. 이 연구의 목적은 표준화된 치주염 모델을 이용하여 개에서 사용할 수 있는 SDD 용량을 확인하는 것이며, 본 논문은 총 3개의 장으로 구성되어있다.

제1장에서는 기존의 ligature induced periodontitis model에 비해, 보다 효율적으로 치주염을 유발하는 방법에 대하여 연구하였다. 비글견 20두를 유발 방법에 따라 네 군으로 나누고 (대조군, 철사 유발군, silk 봉합사 유발군 및 철사와 silk를 꼬아서 적용한 유발군), 군 별로 각기 다른 유발 물질을 개체 당 6개 치경부에 결찰하였으며, 모든 실험군에 반고형식을 급여하면서 치주염을 유발하였다. 유발 방법에 따른 치주염 유발 효율을 확인하기 위해 결찰 이전과 60일 이후의 치주 상태를 평가하여 치주염의 진행 정도를 비교하였으며, 평균 결찰 유지 기간을 비교하였다. 철사와 silk를 꼬아서 적용한 군의 결찰이 다른 유발군에 비해 유의적으로 오래 유지되었으며, 치주염도 유의적으로 심하게 유발되었다 ($P<0.05$).

제2장에서는 개의 치주염에 적용할 수 있는 SDD의 투약 용량을 확인하였다. 20두의 비글견을 각 5두씩 네 군으로 나누어 각각 1, 2, 3 및 5 mg/kg의 용량으로 doxycycline을 1회 투여하였으며, 투여 시점을 기준으로 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 및 24 시간째의 혈중 doxycycline 농도를 고압 액체 chromatography (HPLC)를 이용하여

측정하였다. 실험 결과, 1, 2 mg/kg을 투여한 군의 평균 doxycycline 혈중 농도가 24시간 동안 최저억제농도 이하로 유지됨을 확인하였다. 이러한 방법 의해 증명된 SDD를 한 달 간 비글견에 투약하여 그 효과를 평가하였다. 15두의 비글견을 각 5두씩 세 군으로 나누어 각각 0, 1 및 2 mg/kg의 doxycycline 캡슐을 1일 1회의 용법으로 1개월 간 투약하였으며, 투약 전과 후에 치주 임상 지수를 측정하고 치주조직을 채취하여 zymogram을 실시하였다. SDD를 지속적으로 투여한 실험군에서는 치주 부착 정도 및 치은 출혈지수 등의 임상 지수들이 투여 용량에 비례하여 유의적으로 개선되었으며, 치주조직에서의 MMPs 발현 정도는 대조군에 비해 두 종류의 SDD 군에서 유의적으로 감소하였다 ($P<0.05$).

제3장에서는 SDD의 장기 투여가 치주의 정상 세균총에 미치는 영향을 확인하였다. 12두의 정상 치주를 가진 비글견을 이용하여 제1장에서 기술한 방법으로 치주염을 유발시켰으며, 6두씩 2군으로 나누어 각각 doxycycline 2 mg/kg (SDD) 혹은 placebo capsule을 1일 1회 2개월간 투약하였다. 투약 시작 전, 투약 후 4주 및 8주에 치주 상태의 임상지수를 측정하고 치은하 치태를 채취하여 1주일간 혐기 배양하였다. 배양 후 총 세균 수, *Porphyromonas* spp., *Bacteroides* spp., *Pasturella* spp.의 군집 수를 측정하였으며, 2개월 후 군집 수의 변화를 관찰하여 8주 간의 SDD 투약이 doxycycline의 최소살균농도에 영향을 미치는지 평가하였다. 8주 투약 후 임상지수는 대조군과

비교하였을 때 SDD 군에서 유의적으로 개선되었으며, 총 세균 수에 대한 유의적인 차이는 보이지 않았다. 투약 이후 최소살균농도에서 치은하 세균총을 배양하였을 때 SDD 군과 대조군 모두에서 유의적인 차이가 없음을 확인하였다.

본 연구에서는 개에서 2 mg/kg의 doxycycline을 경구투여할 경우 혈중에서 항생효과를 가지는 농도를 넘지 않는 것을 확인하였다. 또한, 이러한 용량으로 하루 1회 2개월 간 투여하였을 때에 세균의 항생제 감수성에 영향을 미치지 않으면서 치주상태를 개선시키는 것을 확인하였다. 따라서 개의 치주염 치료를 위한 항생농도 이하의 doxycycline은 2 mg/kg, 1일 1회 경구투여가 적합할 것으로 판단된다.

주요어: 항생농도 이하, Doxycycline, 치주염, 개

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