Studies on therapeutic and preventive methods for feline gingivitis.

（猫の歯肉炎に対する治療法および予防法に関する研究）

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Preface

In humans, oral diseases are among the most common health conditions, with periodontal disease standing out as one of the most prevalent and impactful oral diseases globally (Peres, 2019). As periodontal disease progresses, irreversible absorption of periodontal tissues occurs (periodontitis). Lost periodontal tissues do not easily regenerate and cause various symptoms in the organism. Pain, bleeding, halitosis, decreased appetite, and systemic inflammation of organs due to hematogenous dissemination of oral bacteria become significant issues. Periodontal disease may exert an impact on quality of life of individuals, with greater severity of the disease associated with greater impact (Ferreira et al., 2017). Given that the risk of periodontal disease can be mitigated by maintaining good oral hygiene, regular oral care is promoted worldwide (Lertpimonchai et al., 2017).

Similarly, in small animals such as dogs and cats, periodontal disease is the most commonly observed oral pathology, with its pathogenesis, progression, and symptoms considered analogous to those in humans. By two years of age, 70% of cats and 80% of dogs have some form of periodontal disease (Niemiec et al., 2020). However, patients generally exhibit few external clinical symptoms, leading to the initiation of treatment in the

later stages of the disease. In advanced cases of periodontal disease, it can contribute to the progression of secondary pathologies such as pathological mandibular fractures, periapical lesions (suborbital abscess), and tooth resorption, eventually necessitating tooth extraction. Therefore, early intervention in periodontal disease or thorough oral care becomes crucial for animals.

Given that animals cannot comprehend the importance of oral care and many individuals show aversion to having their oral cavity touched, implementing oral care for pets is extremely challenging. In other words, owners need to deepen their understanding of oral diseases and make a conscious effort to control their pet's oral environment. In recent years, there has been an increased awareness among dog owners regarding oral care, facilitated by active guidance from veterinarians, the availability of oral care products, and ease of tooth brushing through training. However, for cats, despite having an awareness of oral hygiene management, it remains challenging to perform tooth brushing due to difficulties in training.

There is a need for feasible and effective treatment and prevention of periodontal disease in cats. The present study therefore focused on gingivitis, the early stage of periodontal disease, and investigated methods to control it (Chapter 1) and causative microorganisms (Chapter2).

Chapter 1

Long-term follow-up study after administration of a canine interferon-α preparation for feline gingivitis

Introduction

Gingivitis is a reversible inflammation of the gingiva caused by oral bacteria in dental plaque. It is the earliest sign of periodontal disease, the most common oral disease seen in cats (Gorrel, 2004). Individuals with untreated gingivitis may develop periodontitis that causes irreversible destruction of the periodontium, including alveolar bone (Gorrel, 2004). Therefore, early treatment or prevention of gingivitis is required in order to avoid periodontitis.

In dogs, it has been shown experimentally that low dose oral administration of canine interferon alpha (CaIFN-α) subtype 4 reduced gingivitis (Ito et al., 2010). Furthermore, it has been reported that CaIFN-α is useful clinically in dogs for oral hygiene control after dental treatment, or from a young age, due to its gingivitis-reducing effect and long-term action (Yamaki et al., 2017). However, the efficacy of CaIFN-α in the treatment of feline gingivitis has not been confirmed.

CaIFN-α is a type I interferon that exhibits immunostimulating, antibacterial, and anti-inflammatory activities in vivo (Dec and Puchalski, 2008; Guarda et al., 2011; Malireddi and Kanneganti, 2013). In dogs, it was reported that the number of *Porphyromonas* spp.

considered to be closely involved in periodontal disease was reduced by intraoral administration of CaIFN-α (Hardham et al., 2005; Ito et al., 2010; Senhorinho et al., 2011). In cats, *Porphyromonas* spp. is also frequently isolated from the oral cavity (Pérez-Salcedo et al., 2013). Based on these findings, it was considered that gingivitis may be reduced by intraoral administration of CaIFN-α not only in dogs, but also in cats.

This study was performed to clarify the clinical usefulness of intraoral administration CaIFN-α in cats. The degree and duration of its effects against feline gingivitis were examined by observing cats administered a CaIFN-α preparation over a long period.

Materials and methods

We randomly selected 13 cats with mild gingivitis visiting our veterinary clinic (Table 1-1). They had not received plaque/calculus removal treatment such as scaling within the previous three months. The cases were divided into two groups: a CaIFN-α administered group (group A: nine cats) and an untreated group (group B: four cats). In addition, we prohibited any diet changes, use of antibiotics or other interferon preparations in these cats, in order to avoid any other influence on the intraoral environment after starting observation.

In group A, InterBerry α® (Hokusan Co., Ltd., Hokkaido, Japan/DS Pharma Animal Health Co., Ltd., Osaka, Japan) which is a lyophilized strawberry powder expressing CaIFN-α subtype 4 by gene recombination was used as the CaIFN-α preparation. For each administration, 0.25 g of a powder preparation containing 250 Laboratory Units of CaIFN-α was suspended in 0.1 ml of water and applied to the entire gingiva with a finger. This treatment was administered once every three days, for a total of 10 times (30 days). This was regarded as one course of treatment. The method of administration and dosage in this study followed those of InterBerry α®.

Observations were performed at the start of CaIFN-α administration (zero months) and at 1, 2, 3, 6, 9 and 12 months after the start of administration. At each observation time point, the degree of gingivitis, plaque/calculus deposition and halitosis were evaluated and scored based on criteria (Table 2, Fig. 1) modified from the previously reported evaluation method (Eubanks, 2010; Miyazaki et al., 1999; Ueda, 1990). Regarding the degree of gingivitis and plaque/calculus deposition, the buccal sides of the left, right, upper and lower canines and carnassials (upper fourth premolar and lower first molar) were examined. Halitosis was organoleptically evaluated by putting the observer's nose to within 10 cm of each cat's open mouth.

The total score for gingivitis and plaque/calculus for each cat were obtained by adding the scores for all eight investigated teeth. Since A-2 lacked one tooth at the starting observation, the total score of this case was expressed as 8/7 times. Cases A-4/A-6 and A-5 were only observed up to six and nine months, respectively, as they were no longer available for observation. For each item, the difference between the scores at zero months and at each observation time point was expressed as the difference value, and statistical analysis was performed for the median of the difference value. Significant differences between at zero months and at each observation time point were determined by Wilcoxon's signed rank sum

test. Significant difference between groups A and B at each observation time point was determined by the Mann-Whitney U test. P values of <0.05 were considered significant.

Results

The changes in the gingivitis score were compared in groups A and B (Fig. 2). In group A, the scores decreased after starting CaIFN-α administration and were significantly lower at one to three months than at zero months. Thereafter, the scores tended to increase, becoming similar to the score at zero months at nine months. In group B, the scores tended to increase, and were higher through all observation time points than the score at zero months. Furthermore, the difference values at one to three months in group A were significantly lower than those in group B.

In addition, cats in group A were divided into two groups according to their total gingivitis score at zero months: One group with scores lower than 10 (group A1; six cats) and the other group with scores higher than 10 (group A2; three cats). The percent change in the total gingivitis score at each observation time point after zero months was expressed as a relative value, and compared among groups A, A1 and A2 (Fig. 3). Significant differences among groups A, A1 and A2 at each observation time point were determined by the Kruskal-Wallis test. Although the score in group A2 decreased more than in the other groups, both groups A1 and A2 showed the lowest values at two or three months, and the transition was similar

to that in group A.

The changes in the plaque/calculus score were compared in groups A and B (Fig. 4). The scores did not decrease in either group A or B. In group A, the scores were significantly higher at six to 12 months than the score at zero months. In group B, the scores increased at 12 months without significant difference. Furthermore, the difference value at six months in group A was significantly higher than that in group B.

The changes in the halitosis score were compared in groups A and B (Fig. 5). In group A, the scores were significantly lower at one to two months than the score at zero months. Thereafter, the scores became similar to the score at zero months. In group B, the score tended to increase without decreasing. There was no significant difference between groups A and B.

Discussion

In this study, gingivitis and halitosis scores decreased only in the CaIFN-α-administered group. Feline gingivitis is caused by a proliferation of bacteria (mainly *Porphyromonas* spp.) in the gingival sulcus (Pérez-Salcedo et al., 2013). Also, halitosis is caused by volatile sulfur compounds produced by these periodontal pathogenic bacteria (Culham et al., 1998). It was speculated that the reduction in gingivitis and halitosis in these cats was caused by a reduction in *Porphyromonas* spp. due to the action of CaIFN-α, as reported in dogs.

It was reported that the gingivitis-reducing effect of CaIFN-α in dogs was maintained up to nine months (Yamaki et al., 2017). However, the duration of the CaIFN-α gingivitis-reducing effect in cats was suggested to be about three months and shorter than that in dogs.

From a comparison within group A, it was found that administration of the CaIFN-α preparation reduced gingivitis at a constant rate, regardless of the degree. In other words, CaIFN-α was at least partially effective against severe gingivitis. However, this also indicates that using CaIFN-α alone is not an adequate treatment for severe gingivitis. For example, it is thought gingivitis with a score of three could not be reduced to a score of zero using CaIFN-

α alone. Therefore, it is necessary that the CaIFN-α preparation be administered after basic treatment for periodontal disease, such as scaling, if cats have severe gingivitis.

Plaque is a biofilm mainly composed of aggregates of bacteria, and calculus is calcified plaque (Gorrel, 2004). Therefore, it was speculated that plaque and calculus are also suppressed by the antibacterial action of CaIFN-α. However, this study showed CaIFN-α may not have a reducing or preventive effect on plaque or calculus. Even so, when the same numbers of specimens with almost the same control score were selected from groups A and B and compared, inhibition of deposition of plaque/calculus by CaIFN-α was suggested. Regarding these effects, further investigation is necessary.

Amimoto et al. (1999) reported that periodontal disease symptoms, including gingivitis, were present in 58.2% of cats even in the first year of life, at which point calculus deposition is low, and that its degree worsened with age. Also, according to the AAHA Dental Care Guidelines in 2013 (Holmstrom et al., 2013), periodontal diseases may accompany various abnormalities in the oral cavity from birth to two years of age in cats. Furthermore, the WSAVA Global Dental Guidelines in 2017 (Niemiec et al., 2017), reported that 70% of cats have some form of periodontal disease by two years of age. Recently, to control oral hygiene before

periodontal disease becomes severe or after dental treatment such as dental calculus removal, as well as efforts to prevent periodontal disease, have been regarded as important. Although tooth brushing is the most recommended approach to oral hygiene control, it is difficult to perform in cats. Therefore, a CaIFN-α preparation that can be administered intraorally with relative ease, may useful for oral hygiene control in young cats or after dental treatment.

Table 1. Details of cats observed in this study

Group	No.	Age(years)	Breed	Sex	Weight (kg)	Remarks
A	1	10.0	DSH	FS	4.9	
	2	8.0	DSH	FS	3.5	Missing upper left canine tooth
	3	5.0	DSH	FS	4.0	
	4	2.3	DSH	FS	4.6	Observed up to 9 months
	5	8.4	DSH	MC	5.4	Observed up to 6 months, FCV(+)
	6	4.1	DSH	MC	4.8	Observed up to 9 months, FeLV(+)
	7	4.0	Maine Coon	MC	4.2	
	8	1.0	DSH	FS	2.7	
	9	2.7	DSH	FS	3.1	FIV(+)
B	1	3.0	DSH	MC	4.5	
	2	0.8	DSH	FS	4.3	
	3	6.8	DSH	FS	4.5	
	4	4.8	DSH	FS	3.2	

Each cat visited our hospital on routine examinations and was diagnosed mild gingivitis. Then, they were classified into canine interferon alpha administered group A and untreated group B and were observed the progress. DSH: domestic shorthair (the crossbreed cat); FS: female, spayed; MC: male, castrated; FCV: feline calicivirus; FeLV: feline leukemia virus; FIV: feline immunodeficiency virus.

Table 2. Evaluation criteria and score for each item

Score	Evaluation criteria		
	Gingivitis	Plaque / Calculus	Halitosis
0	No gingival inflammation	No plaque or calculus deposition	Odorless
0.5	Slight gingival inflammation (slight change in color)	–	–
1	Mild gingival inflammation (clear redness and edema; no bleeding on probing)	1/3 or less of the crown buccal surface covered	Smell, but no malodor
2	Moderate gingival inflammation (strong redness and edema; bleeding on probing)	1/3-2/3 of the crown buccal surface covered	Faint malodor
3	Severe gingival inflammation (marked redness and edema; ulceration; tendency to spontaneously bleed)	2/3 or more of the crown buccal surface covered	Definite malodor
4	–	–	Bearable, strong malodor
5	–	–	Unbearable, intensive malodor

Fig. 1. Gingival appearance for each gingivitis score evaluation criteria. All images show the buccal gingival margin of the upper right canine. Scores were determined by gingival color, edema, bleeding, etc.

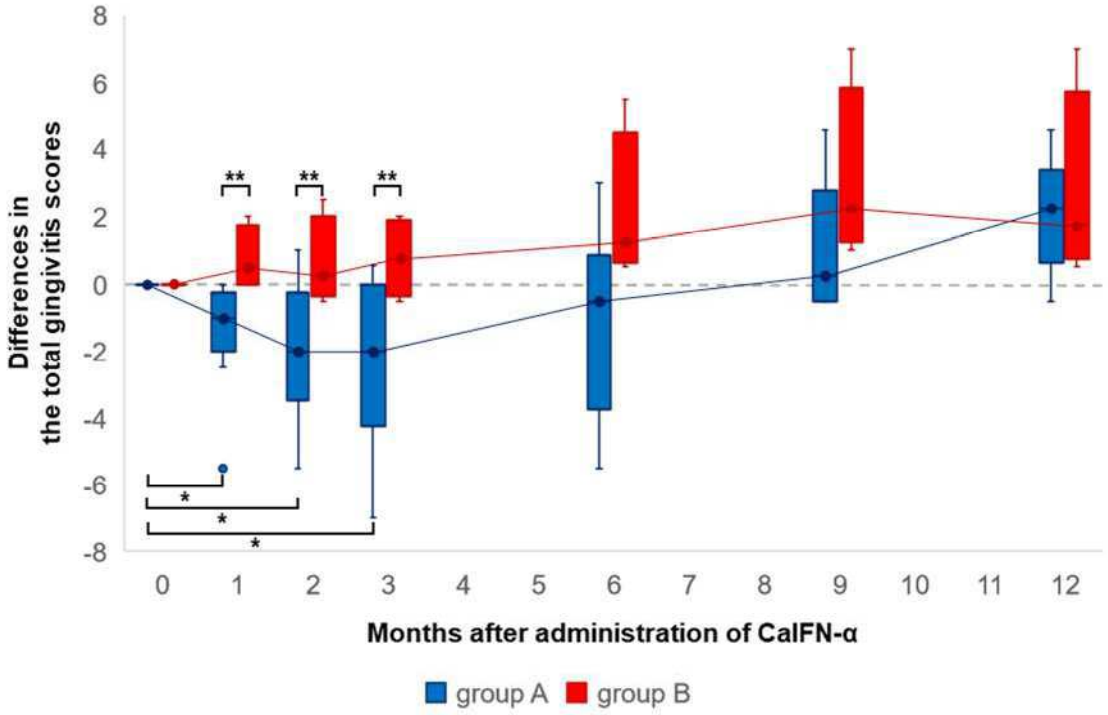

Fig. 2. Boxplots showing differences at each observation time point from zero months regarding the total gingivitis score in groups A and B. Group A (nine cats) was administered canine interferon alpha (CaIFN-α). Group B (four cats) was not administered CaIFN-α. The line with a point within the box represents the median, and the lower and upper lines of the box represent the 25th and 75th percentiles (interquartile range, IQR). Whiskers represent the maximum and minimum values within 1.5 times the IQR. The point outside the whiskers is an outlier. "*" indicates significant difference at each observation point from zero months ($P<0.05$). "**" indicates significant difference between groups A and B ($P<0.05$).

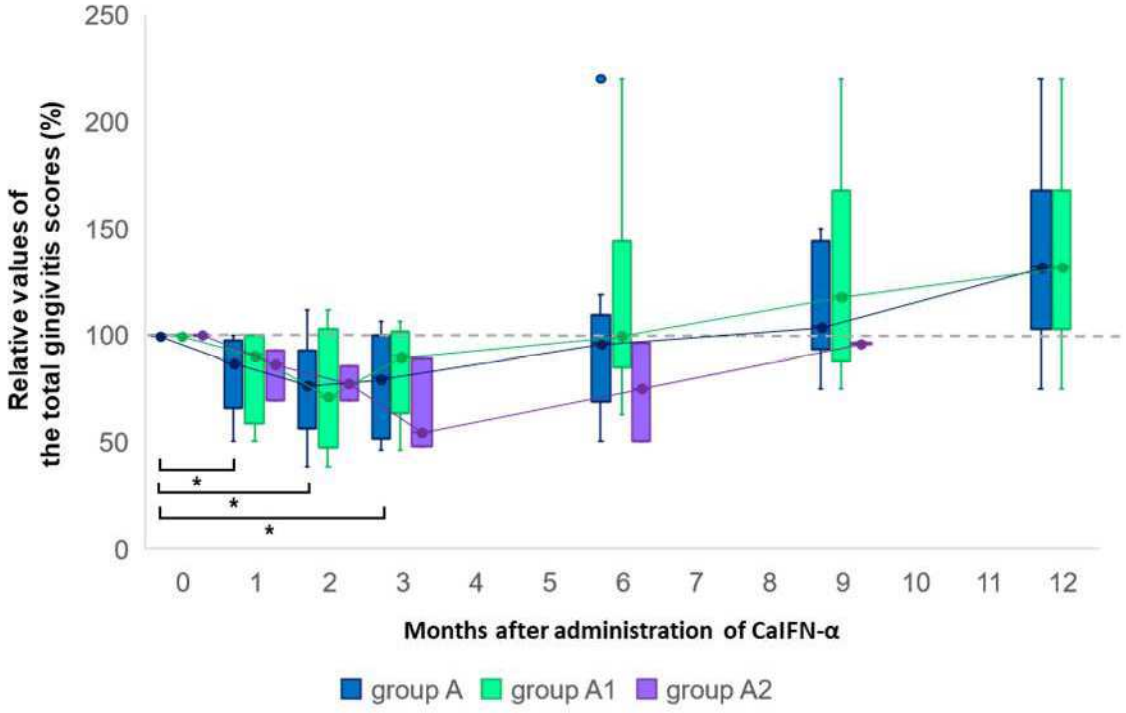

Fig. 3. Boxplots showing percent changes at each observation time point from zero months regarding the total gingivitis score in groups A, A1 and A2. Group A (nine cats) was administered canine interferon alpha (CaIFN-α). Cats in group A were divided into two groups according to their total gingivitis score at zero months: group A1 (six cats) with a score lower than 10 and group A2 (three cats) with a score higher than 10. "*" indicates significant difference at each observation point from zero months ($P<0.05$). No significant difference among groups A, A1 and A2 at all observation points ($P<0.05$).

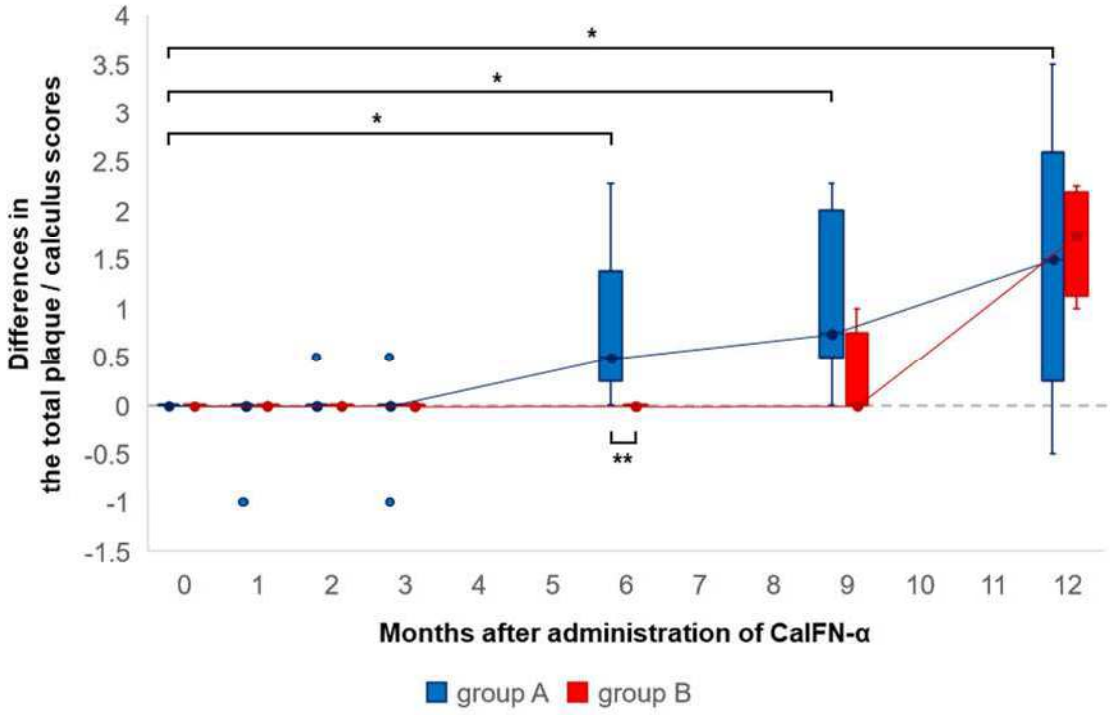

Fig. 4. Boxplots showing differences at each observation time point from zero months regarding the total plaque / calculus score in groups A and B. Group A (nine cats) was administered canine interferon alpha (CaIFN-α). Group B (four cats) was not administered CaIFN-α. "*" indicates significant difference at each observation point from zero months ($P<0.05$). "**" indicates significant difference between groups A and B ($P<0.05$).

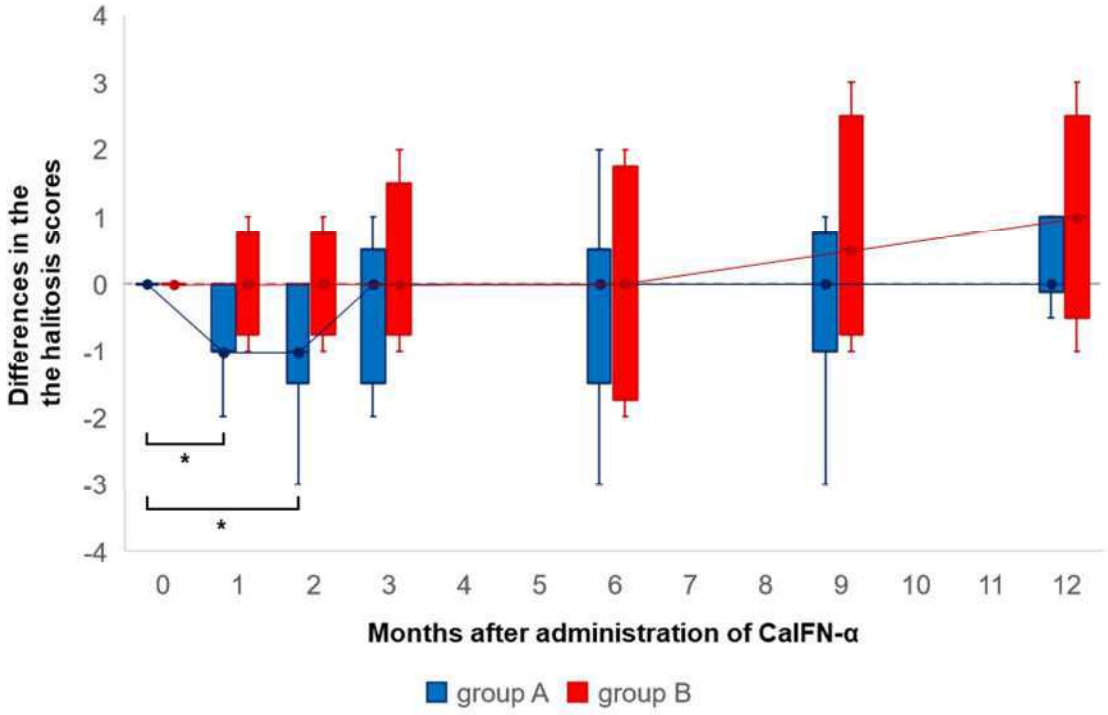

Fig. 5. Boxplots showing differences at each observation time point from zero months regarding the halitosis score in groups A and B. Group A (nine cats) was administered canine interferon alpha (CaIFN-α). Group B (four cats) was not administered CaIFN-α. "*" indicates significant difference at each observation point from zero months ($P<0.05$). No significant difference between groups A and B at all observation points ($P<0.05$).

Connecting Chapter

It had been confirmed that oral administration of CaIFN-α exhibits a reduction effect on gingivitis in cats. In other words, CaIFN-α is an effective therapeutic agent for cats prone to developing gingivitis. Moreover, due to its minimal side effects and the possibility of continuous administration, it can be utilized for the prevention of periodontal disease.

Generally, *Porphyromonas* spp. are mainly involved in periodontal disease in dogs and cats (Senhorinho et al., 2011, Pérez-Salcedo et al., 2013). Oral administration of CaIFN-α in dogs is believed to reduce the number of *Porphyromonas* spp. in saliva, thereby alleviating the symptoms of gingivitis (Ito et al., 2010). On the other hand, in cats, factors associated with the onset and progression of periodontal disease may differ from those in dogs. This is suggested by the shorter duration of the effect of CaIFN-α in cats compared to that in dogs as well as the higher incidence and severity of gingivitis from a young age in cats.

To explore preventive and therapeutic methods for gingivitis in cats, it is essential to gain a more detailed understanding of the mechanisms specific to gingivitis in cats. Therefore, as a starting point, I investigated the pathological factors associated with gingivitis in young cats

from a bacteriological perspective.

Chapter 2

The association between gingivitis and oral spirochetes in young cats and dogs

Introduction

Gingivitis is a reversible inflammation confined to the gingiva, and it is described as an initial stage of periodontal disease (Niemiec et al., 2020). Periodontal disease is a common disease in cats and dogs (Lund et al., 1999, Robinson et al., 2016) and is thought to be initiated by oral bacteria in the plaque that adhere to the teeth. Recent studies have revealed a large diversity of bacterial species in the subgingival plaque of cats and dogs. There are also extensive differences between the microbiome identified in companion animals and humans (Dewhirst et al., 2010, Dewhirst et al., 2012, Dewhirst et al., 2015). Although healthy animals of the same species have similar composition of the oral microbiome, it changes with periodontal disease (Santibáñez et al.,2021).

In dogs and cats, the incidence and severity of periodontal disease, especially periodontitis, are known to increase with age (Harvey et al, 1994, Gengler et al., 1995). Conversely, although periodontitis is rarely seen in dogs or cats under one year old, gingivitis is a common diagnosis. Previous epidemiological studies showed that dogs aged between 0.5 and 1.5 years had no gingivitis (Isogai et al., 1989) whereas approximately 50% of cats below one year of age have established gingivitis (Verhaert and Wetter, 2004). Thus, it is

expected that the prevalence of gingivitis in young cats is higher than that in young dogs.

In humans, many bacterial species have been associated with the diagnosis of periodontal disease. Among them, *Treponema denticola*, a type of spirochete, belongs to the red complex species related to periodontal disease because of its virulence factors, along with *Tannerella forsythia* and *Porphyromonas gingivalis* (Socransky et al., 1998, Ishihara, 2010). Conversely, in dogs and cats, *Porphyromonas gulae* has been found to be the predominant species leading to periodontal disease (Fournier et al., 2001, Kato et al., 2011, Pérez-Salcedo et al., 2013), and research focusing on the role of spirochete is limited. However, recent reports have shown that the plaque from mild feline periodontitis contained a much larger number of *Treponema* species than those in dogs' plaque (Davis et al., 2013, Harris et al., 2015).

We speculate that there may be an association between the high incidence of gingivitis in cats of an early age and the high incidence of spirochetes in cats with mild periodontal disease. Therefore, this study assessed the difference in the prevalence of gingivitis between young cats and dogs, clarifying the relationship between gingivitis and oral spirochetes in young cats.

Materials and methods

Subjects and ethical statement

Examination and sampling of the subjects were performed between April 2020 and March 2022 at Amica Pet Clinic, Yamaguchi, Japan. Cats and dogs younger than one year old were randomly examined. Cats and dogs were excluded from this study as samples if they had systemic underlying diseases that affect the oral cavity or oral diseases other than periodontal inflammatory diseases, or antibiotic treatment in the past month. Dental plaque was sampled and the oral cavity was checked, when the animals were anesthetized. Subgingival and supragingival samples were collected from all 99 animals.

Prospectively, the Yamaguchi University Ethical Committee on Animal Research counseled that because samples were obtained as a part of medical treatment, an Ethical Committee on Animal Research protocol was not required. All owners had given their written consent prior to participating in the study. No adverse events resulting from tissue acquisition were documented.

Clinical evaluation of subjects

Gingiva was evaluated based on the clinical characteristics of the Gingival Index (GI)

system (Löe and Silness, 1963), classified in four levels from zero to three (Fig 1 and Table 1). The same evaluator determined the GI values for all samples. When determining only the presence or absence of gingivitis, it was regarded that GI 1–3 was positive and GI 0 was negative.

Sample collection and preparation of examination

One site of the carnassials (maxillary fourth premolars or mandibular first molars) with the strongest gingival inflammation was targeted. A microbrush (TPC Disposable Micro Applicators Fine) was rubbed firmly five times on the gingival sulcus and the adjacent enamel of the buccal surface of the targeted tooth of each animal.

A part of the plaque sample attached to the microbrush was applied to a new, uncontaminated glass slide and stained with Hemacolor® (Merck, Germany) for microscopic examination. The remaining sample was placed in a tube containing 500 μL of phosphate-buffered saline (PBS) for DNA preparation. PBS samples were stored at −4°C until DNA extraction.

DNA was extracted and purified using a DNeasy Blood & Tissue Kit (QIAGEN, Netherlands) according to the manufacturer's protocol for the polymerase chain reaction (PCR) method. DNA samples were stored at −30°C until needed.

16S ribosomal RNA gene PCR amplification

The specific sequence of the 16S ribosomal RNA gene was amplified using AGAGTTTGATCCTGGCTCAG and GTTACGACTTCACCCTCCT primers selective for the phylum Spirochaetes (Siqueira and Rôças, 2004) or TTGCTTGGTTGCATGATCGG and GCTTATTCTTACGGTACATTCACA primers selective for *P. gulae* (Kato et al., 2011).

PCR was performed using GoTaq® Green Master Mix (Promega, USA) with the following cycling parameters: an initial denaturation at 95°C for 2 min, followed by 25 cycles of 30 s at 95°C, 30 s at 60°C, and 90 s at 72°C for the phylum Spirochaetes or 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for *P. gulae*, with a final extension at 72°C for 5 min. The purity of the product was determined by electrophoresis in a 1% agarose gel using Mupid-2plus (Takara, Japan). DNA was stained with ethidium bromide and viewed under long-wavelength ultraviolet light using a UV transilluminator (ATTO, Japan).

Statistical analysis

Data were analyzed using the software EZR 4.0.3 (Windows 11). Fisher's exact test was used to compare the prevalence of gingivitis or positive rate of bacteria between cats and dogs, considering $P < 0.05$ as statistically significant. In addition, the association between

the clinical characteristics of the gingiva and the detection of bacteria was evaluated by odds ratio (OR), with 95% confidence intervals (95% CI).

Results

Study population

A total of 68 cats (34 males and 34 females) and 31 dogs (14 males and 17 females) were sampled. The age of the cats varied between 5 and 12 months (mean, 7.1 months; SD, 1.4 months), and their weights ranged from 2.04 to 4.66 kg (mean, 3.22 kg; SD, 0.55 kg). The age of the dogs varied between 5 and 11 months (mean, 6.7 months; SD, 1.2 months), and their weights ranged from 1.44 to 27.0 kg (mean, 5.50 kg; SD, 3.34 kg). Further information, including sampling sites and GI of each animal, is shown in S1 and S2 Tables.

Prevalence of gingivitis

The prevalence of gingivitis was 92.6% (63/68) in cats and 45.2% (14/31) in dogs (Fig 2). Cats had a significantly higher prevalence of gingivitis than dogs ($P < 0.05$).

Microscopic examination

To confirm if there were spirochetes in the dental plaque, the samples were stained and observed under an optical microscope. Several types of spirochetes were found based on their morphology (staining, spiral, and width) (Fig 3). At least one key morphological

characteristic (low stainability, many spirals, and small width) was common to almost all spirochete-positive samples. The results were assessed by the presence or absence of spirochete (Table 2). Spirochetes were observed in samples of 51 of 63 cats (81.0%) and 2 of 14 dogs (14.3%) with gingivitis. Spirochetes were observed in samples from 2 of 5 cats (40.0%) and 1 of 17 dogs (5.9%) without gingivitis (Table 2).

Molecular analysis

The results of the PCR for the phylum Spirochaetes are shown in Table 2. A total of 53 of 63 (84.1%) samples from cats with gingivitis and 2 of 5 (40.0%) samples from cats without gingivitis were positive. A total of 3 of 14 (21.4%) samples from dogs with gingivitis and 3 of 17 (17.7%) samples from dogs without gingivitis were positive. Among animals with gingivitis, the detection rate of spirochete was significantly higher in cats than in dogs.

Only 1 of 53 microscopy positive cat samples and 1 of 3 microscopy positive dog samples were PCR negative and 3 of 15 microscopy negative cat samples and 4 of 28 microscopy negative dog samples were PCR positive for the phylum Spirochaetes (Table 3).

The PCR results for *P. gulae* are shown in Table 2. A total of 39 of 63 (61.9%) samples from cats with gingivitis and 2 of 5 (40.0%) samples from cats without gingivitis were PCR positive. A total of 2 of 14 (14.3%) samples from dogs with gingivitis and 4 of 17 (23.5%)

samples from dogs without gingivitis were PCR positive. Among animals with gingivitis, the detection rate of *P. gulae* was significantly higher in cats than in dogs, as was the identification of spirochetes.

The association between the development of gingivitis in young cats and dogs and each bacterial species was examined by OR and 95% CI (Table 4). In young cats, spirochetes (OR = 7.95; 95% CI = 1.17, 53.83; P < 0.05) were shown to be significantly associated with gingivitis, but *P. gulae* (OR = 2.44; 95% CI = 0.38, 15.66; P = 0.23) was not associated with gingivitis. Conversely, in young dogs, neither spirochetes (OR = 1.27; 95% CI = 0.21, 7.58; P = 0.34) nor *P. gulae* (OR = 0.54; 95% CI = 0.08, 3.51; P = 0.29) was associated with gingivitis.

Discussion

To the best of our knowledge, this study reported for the first time a higher prevalence of gingivitis in young cats under one year old by comparing to young dogs. Because gingivitis in small animals presents with few clinical signs, it can only be diagnosed as a pathology with careful observation within the oral cavity. The fact that many veterinarians and owners are unaware of the signs of gingivitis in young animals may have contributed to underdiagnosing this disease.

Periodontal disease is the most common and important health problem in cats and dogs. Gingivitis is the initial, reversible, and preventable stage of periodontal disease, and it may develop to periodontitis, involving the progressive and irreversible destruction of the periodontal tissues (Bellows et al., 2019, Niemiec et al., 2020). Complications include chronic ulcerative paradental stomatitis, faucitis, and chronic gingivostomatitis (Maciel et al., 2020). Therefore, it is desirable to swiftly diagnose gingivitis cases. The 2019 AAHA Dental Care Guidelines for Dogs and Cats (Bellows et al., 2019) recommends that a true dental prophylaxis starts at one year of age for cats and small- to medium-breed dogs and by two years of age for larger-breed dogs, even if there are no obvious lesions. The clarification of

the association between gingivitis in young cats and oral bacteria should help in establishing effective prevention and providing treatment.

Various forms of spirillum have been confirmed in the plaque of dogs and cats at the stage of preliminary experiments, in addition to Treponema, which is usually detected in the oral cavity of humans. Nonetheless, other spirochetal species (such as *Borrelia* and *Brachyspira*) may coexist. Therefore, in this study, primers specific to the phylum Spirochaetes were used to detect spirochetes. The results found by PCR were well correlated with the pictures obtained by microscopy. Because one similar morphology was common to almost all spirochete-positive samples under light microscopy, PCR-positive results were considered to reflect the presence of a particular species.

In one sample, spirochetes were detected under a microscope but not by PCR for both cats and dogs. This may be due to both samples presenting a small number of spirochetes on the smear; the number of bacteria in the sample may be below the detection limit for PCR. It was also possible that there were novel spirochetes that did not match the primer pair used (Valdez et al., 2000) or that an inhibitor was present in the sample.

It is reported that that the microbiota of periodontally healthy cats were distinguishable from diseased cats (Rodrigues et al., 2019). In this study, we showed that spirochetes are more associated with gingivitis in young cats than with *P. gulae*. *P. gulae* is thought to be associated with periodontal disease in cats, and a correlation has been reported between the proportion of this species and the severity of periodontal disease (Pérez-Salcedo et al., 2013). However, because the subjects of the study were the animals with gingivitis (mild periodontal disease), the association of *P. gulae* to the disease may not be significant.

Many studies reported that spirochetes as a group and *T. denticola* are associated with periodontal disease, especially periodontitis in humans (Ellen and Galimanas, 2005). One of the reports showed that *T. denticola* increases susceptibility to gingivitis (Riviere and DeRouen, 1998). On the other hand, only a few studies have investigated the association of oral spirochetes with periodontal disease in animals. However, in recent years, some studies of the subgingival microbiota using next-generation sequencing have found spirochetes to have a higher abundance in periodontally diseased cats compared to healthy (Davis et al., 2013, Rodrigues et al., 2019). Moreover, if there might be more diverse oral spirochetes in dogs and cats than in humans as previous report have shown (Valdez et al., 2000), it is also possible that there are spirochetes with etiologies that are not common in humans. The

association of spirochetes with gingivitis in young cats shown in this study suggests that spirochetes, together with other oral microbes or alone, may play some role in the early stages of periodontal disease in cats.

Fig 1. Examples of gingivitis determination according to the criteria for the GI systems in cats and dogs. The right maxillary fourth premolars (108) are compared.

Table.1 Criteria for the Gingival Index (GI) Systems

GI	Inflammation—Appearance
0:	Absence of inflammation.
1:	Mild inflammation—slight change in color and little change in texture.
2:	Moderate inflammation—moderate glazing, redness, oedema, and hypertrophy. Bleeding on pressure.
3:	Severe inflammation—marked redness and hypertrophy. Tendency to spontaneous bleeding. Ulceration.

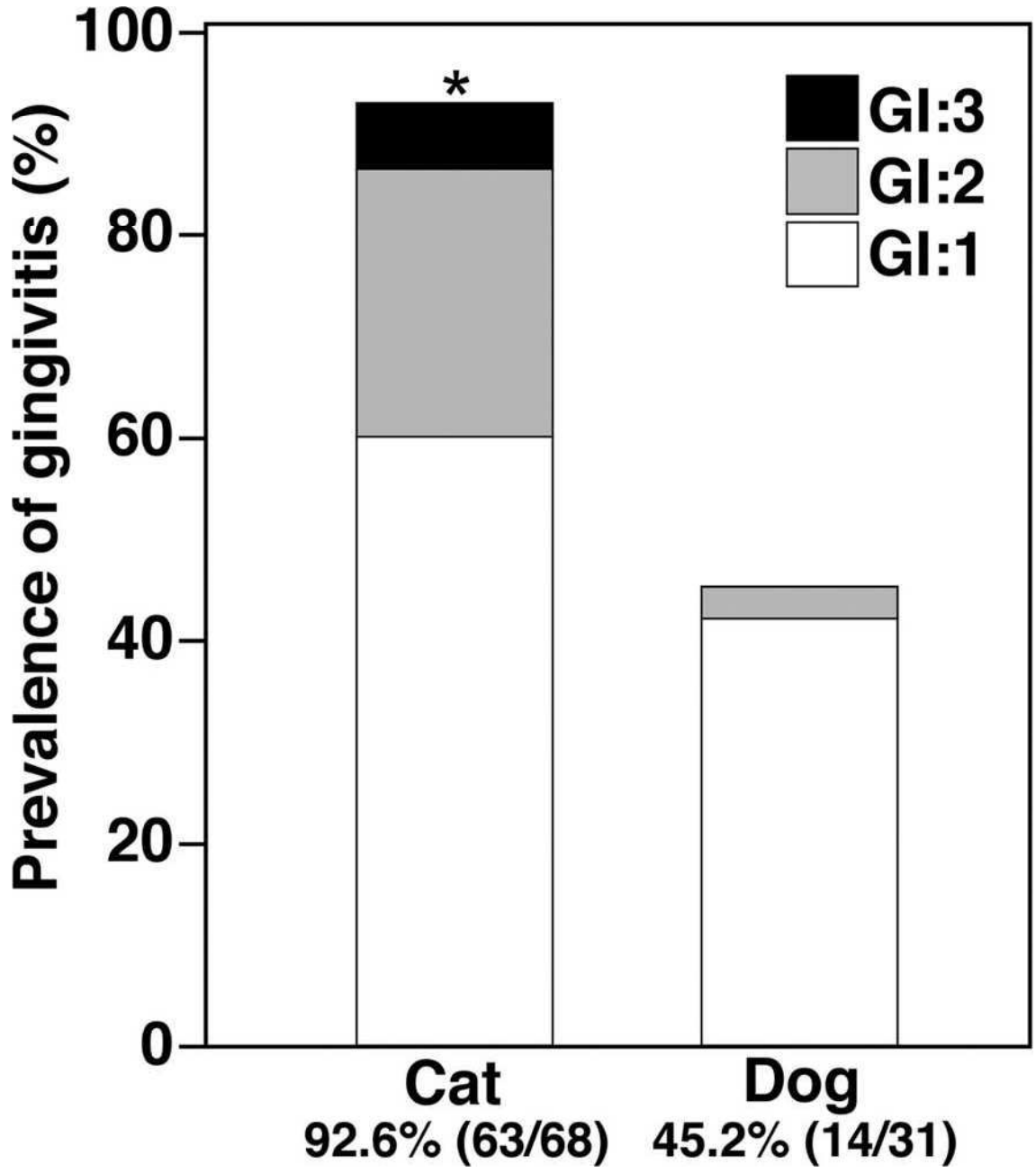

Fig 2. Prevalence of gingivitis in cats and dogs one year old or younger. Significant differences are indicated by asterisks (*P < 0.05).

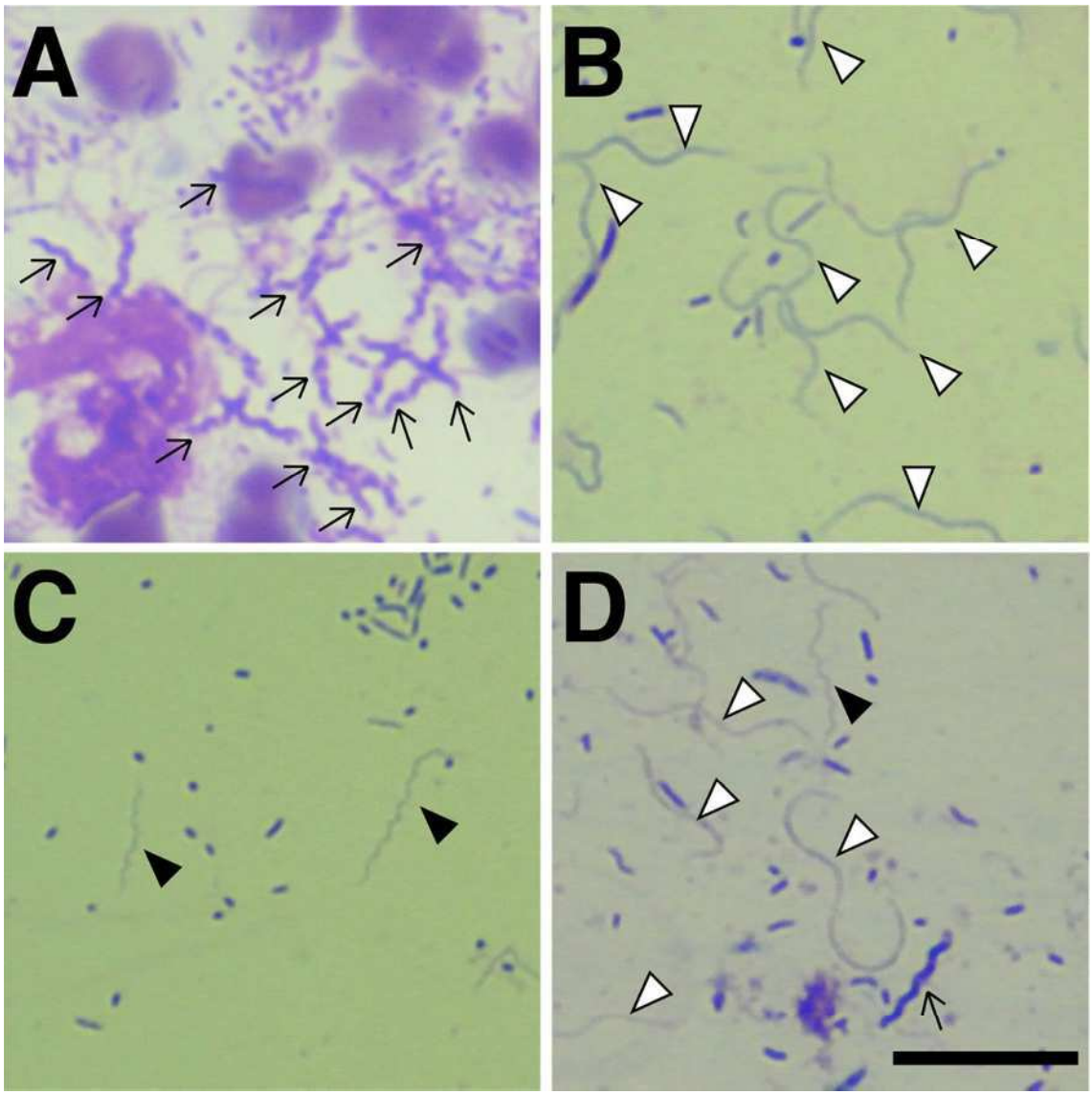

Fig 3. Several morphological types of spirochetes found in dental plaque. (A) High stainability and many spiral type (arrows). (B) Low stainability and little spiral type (white arrowheads). (C) Low stainability and many spiral type (black arrowheads). (D) Many types of spirochetes. Scale bar represents 10 μm.

Table. 2. Rate of positivity for spirochetes and *P. gulae* by microscope or PCR examination

	N	Microscope	PCR	
		Spirochetes (%)	Spirochetes (%)	*P. gulae* (%)
Cat	68			
with Gingivitis	63	51 (81.0)	53 (84.1)	39 (61.9)
without Gingivitis	5	2 (40.0)	2 (40.0)	2 (40.0)
Dog	31			
with Gingivitis	14	2 (14.3)	3 (21.4)	2 (14.3)
without Gingivitis	17	1 (5.9)	3 (17.3)	4 (23.5)

Table. 3. The correlation between positive rates of spirochete by PCR or microscopic examination

Cat		PCR (%)	
		+	−
Microscope (%)	+	52 (76.47)	1 (1.47)
	−	3 (4.41)	12 (17.65)

Dog		PCR (%)	
		+	−
Microscope (%)	+	2 (6.45)	1 (3.23)
	−	4 (12.90)	24 (77.42)

Table. 4. Odds ratio of Spirochaetes or *P. gulae* for gingivitis.

		Odds Ratio	95% CI	*P* value
Cat				
	The Phylum Spirochaetes	7.95	1.17-53.83	<0.05
	P. gulae	2.44	0.38-15.66	0.23
Dog				
	The Phylum Spirochaetes	1.27	0.21-7.58	0.34
	P. gulae	0.54	0.08-3.51	0.29

Conclusion

As gingivitis progresses, it can shift to irreversible periodontitis, leading to a reduced quality of life. Therefore, early treatment and prevention of periodontal disease in animals is crucial, but oral care in cats is often particularly difficult. Oral hygiene management measures that can be implemented in cats need to be considered. In addition, an understanding of factors related to the onset and progression of gingivitis is required in order to effectively implement treatment and prevention. Against this background, the effectiveness of CaIFN-a in inhibiting the progression of gingivitis in cats was demonstrated in Chapter 1 and the high prevalence of gingivitis and the association of spirochetes with gingivitis in young cats in Chapter 2.

Considering preventive and therapeutic measures from a young age or in the early stages of periodontal disease, examination of oral spirochetes can be an important risk marker for periodontal disease. In addition, treatment of spirochetes can be an effective approach to prevent the progress of periodontal disease. To strengthen these possibilities, it is necessary to confirm that a reduction in oral spirochetes actually leads to a reduction in gingivitis symptoms. Although it is evident that oral administration of CaIFN-α can reduce the number

of *Porphyromonas* spp. in saliva, its efficacy against spirochetes remains unclear. Further studies are necessary to clarify the relationship between CaIFN-α and spirochetes.

Periodontal disease is a multifactorial inflammatory disease that develops and progresses under the influence of bacteria, their products and host responses (Niemiec et al., 2020). In addition, CaIFN-α is a type I interferon that exhibits immunostimulating, antibacterial and anti-inflammatory activities *in vivo* (Dec and Puchalski, 2008; Guarda et al., 2011; Malireddi and Kanneganti, 2013). The reduction in gingivitis observed following a low dose oral administration of CaIFN-α to cats may be attributed to its multilateral activities. CaIFN-α preparations that actually provide symptomatic relief and are easily and sustainable administered may be useful in the treatment and prevention of gingivitis in cats.

As cats age, the prevalence and severity of periodontal disease tend to increase (Gengler, 1995). Surgical scaling or tooth extraction requiring anesthesia becomes applicable for severe periodontal lesions. However, there may be cases where surgery cannot be performed due to underlying conditions or age-related factors when treatment is deemed necessary. Similar to humans, in cats, there is a need to place greater importance on the prevention or early treatment of periodontal disease.

Overall, this study demonstrates the utility of spirochetes as a risk factor and CaIFN-α as an effective treatment for cats with periodontal disease. I hope that my research contributes to enhancing the quality of life for cats and, ultimately, improving the well-being of other companion animals and humans.

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