Short Communication

First application of Chitin nanofiber gel for feline gingivitis: a pilot study

Hinako Harada[1] Yuuka Kanao[1] Kazuki Harada[1*]

Abstract

Gingivitis is a highly prevalent, refractory disease in cats. Chitin nanofibers (CNFs) have clinical efficacy against chronic periodontitis in humans but this remains to be clarified in cats. In this study, we examined the effect of CNFs on feline gingivitis. A gel containing CNF was orally applied to seven cats with spontaneous gingivitis once a day for 1 month. Gingivitis, plaque/calculus deposition and halitosis were evaluated before and after treatment. We also investigated changes in subgingival microbiota using next-generation sequencing analysis. After CNF treatment, the median total gingivitis score was reduced significantly ($P = 0.0431$). Plaque/calculus and halitosis scores in several cats decreased but did not change significantly between before and after treatment (both $P = 0.1088$). Subgingival microbiota analysis revealed that the relative abundance of Absconditabacteriales, Aquaspirillum and Phaselicystis significantly increased after treatment compared with before treatment ($P < 0.05$). In addition, the relative abundance of Clostridia_UCG-014 and Peptostreptococcus marginally decreased and that of Saccharibacteria, Dojkabacteria, and Arcobacter spp., marginally increased from before to after treatment ($P < 0.10$). Our results provide some support for the use of CNF as one of the treatment options for feline gingivitis.

Keywords: cats, chitin nanofibers, gingivitis, subgingival microbiota

[1]*Laboratory of Veterinary Internal Medicine, Joint Department of Veterinary Medicine, Faculty of Agriculture, Tottori University, Japan*

****Correspondence**: k-harada@tottori-u.ac.jp* (K. Harada)

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Introduction

Gingivitis is an inflammatory disease that is highly prevalent in cats and is characterized by red or swollen gingiva (or both), halitosis and an accumulation of plaque or calculus (or both) on the teeth and gingiva (Niemiec, 2008; Perry and Tutt, 2015). Severe feline gingivitis can also be painful and affected cats have difficulty eating, which can adversely affect their quality of life (Niemiec, 2008; Perry and Tutt, 2015). The presence of subgingival bacterial plaque, which leads to marginal tissue inflammation in the gingiva, is considered to be the main cause of feline gingivitis (Rodrigues *et al.*, 2019). The basic treatment for feline gingivitis includes the use of antimicrobial or immunosuppressive drugs (or both), scaling and polishing of teeth and, in severe cases, the removal of teeth as sources of inflammation (Perry and Tutt, 2015; Williams and Aller, 1992). Nevertheless, drug treatments are accompanied by the risk of adverse effects (e.g., the development of antimicrobial-resistant bacteria) and the latter two treatments necessitate general anesthesia. Thus, the development of a safer treatment of feline gingivitis would be desirable.

Chitin nanofibers (CNFs) have been developed from mechanically and chemically disintegrated chitin, poly[β-(1-4)-*N*-acetyl-D-glucosamine], the second most abundant biopolymer after cellulose, for medical purposes (Ifuku and Saimoto, 2012). Previous studies have demonstrated that chitin possesses various beneficial properties, including anti-inflammatory activity (Azuma *et al.*, 2014), bacteriostatic activity (Benhabiles *et al.*, 2012; Raut *et al.*, 2016), and activation of microbiota (Azuma *et al.*, 2015), as well as biocompatibility and biodegradability (Zhu *et al.*, 2019). Additionally, chitosan, a derivative of chitin, has an antibiofilm effect on periodontal pathogens (Costa *et al.*, 2014) and clinical efficacy against chronic periodontitis (Akincibay *et al.*, 2007) in humans. Thus, CNF may be an effective treatment of feline gingivitis, but the effects of CNF on the disease have not previously been investigated.

Next-generation sequencing (NGS) technique, differs from conventional bacterial identification in that it enables us to sequence hundreds of genes at once and has a deeper coverage of the microbial community (Zhang *et al.*, 2021). On this background, there have been many researches utilizing NGS for oral microbiota in human medicine (Zhang *et al.*, 2021) and veterinary medicine (Rodrigues *et al.*, 2019; Oba *et al.*, 2022). In this study, we evaluated the clinical scores of gingivitis, subgingival microbiome and adverse reactions in cats treated with CNF gel to assess its performance and safety.

Materials and Methods

The animal experiment in this study was conducted under an ethics committee-approved protocol in accordance with the Tottori University Animal Use Committee (approval number 21-T-3). Seven mixed-breed cats (four males and three females; mean age 3.9 ± 0.4 years and bodyweight 2.8–4.8 kg) were subjected to this study. All the cats were diagnosed with pre-existing inflammation of the gingiva (gingivitis) but not periodontitis (no loss of attachment) by probing with a hand scaler. Additionally, physical and blood examinations confirmed none of the cats had infectious or systemic diseases, including feline leukemia virus, feline immunodeficiency virus, severe kidney disease and diabetes mellitus. All the cats had been given the same commercial food and no other treatment during the study period. Marine Nano-fiber Oral care gel for animals (Marine Nanofiber Co., Ltd., Tottori, Japan), which is 0.7 g of gel containing partially deacetylated CNF, in addition to water, glycerin and lactic acid, was orally applied by spontaneous lick in all cats once a day at least 1 h after eating during the 1-month study period.

Observations were performed before the start of CNF administration. The degrees of gingivitis, plaque/calculus deposits and halitosis were evaluated and scored according to previously reported criteria (Yamaki *et al.*, 2020). Briefly, the gingivitis scores were evaluated as 0 (No inflammation), 0.5 (Slight inflammation), 1 (Mild inflammation), 2 (Moderate inflammation) and 3 (Severe inflammation), as shown in Fig. 1. The plaque/calculus scores were evaluated as 0 (No plaque or calculus), 1 (1/3 or less of crown surface covered), 2 (1/3-2/3 of crown surface covered), and 3 (2/3 or more of crown surface covered). In addition, halitosis scores were evaluated as 0 (Odorless), 1 (Smell, but no malodor), 2 (Faint malodor), ,3 (Definite malodor), 4 (Strong malodor), and 5 (Intensive malodor). The upper buccal sides of the left and right canines and molars were examined for gingivitis and plaque/calculus, and all the scores were added to obtain total scores. To evaluate halitosis, the observer sniffed by placing the nose within 10 cm of each cat's open mouth (Yamaki *et al.*, 2020).

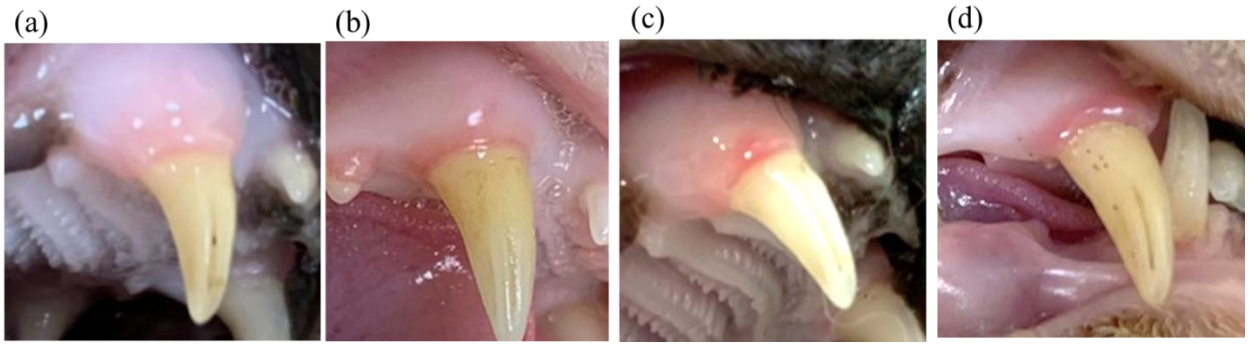


Figure 1 Appearance of feline gingiva with gingivitis scores of 0 (a), 0.5 (b), 1 (c), and 2 (d). The cat with gingivitis score of 3 was not subjected to this study.

Bacterial samples were obtained from a canine tooth of each cat by the established technique of using a sterile endodontic paper point (Pérez-Salcedo *et al*., 2011) at pretreatment observation. The sequencing and sequence analysis of bacterial 16S rRNA gene amplicon was outsourced to Anicom Pafe, Inc. (Tokyo, Japan), according to the previous study (Mizukami *et al*., 2019). Briefly, genomic DNA was extracted using CheMagic DNA Stool kit (PerkinElmer Inc., MA, USA) for automated DNA purification on the CheMagic 360 (PerkinElmer). Each PCR product was purified using a Sera-Mag Select (Cytiva, Tokyo, Japan) and quantified using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) (Mizukami *et al*., 2019). Amplification of the V3-V4 region of 16S rRNA gene was performed by PCR using the S-D-Bact-0341-b-S-17 and the S-D-Bact-0785-a-A-21 primers (Klindworth *et al*., 2013) attached with each Illumina overhanding adapter sequence. Second PCR was conducted for indexing, using the Nextera XT index kit set v2 (Illumina Inc., CA, USA), according to the manufacture's protocol. Pair-end sequencing was carried out using Illumina MiSeq with a MiSeq Reagent Kit v3 (600 cycle; Illumina) (Mizukami *et al*., 2019). The sequence data was analyzed using QIIME 2 (Quantitative Insights into Microbial Ecology 2) v 2020.6.0. Each amplicon sequence variant was taxonomically classified using the SILVA 138 SSU database (Yilmaz *et al*., 2014).

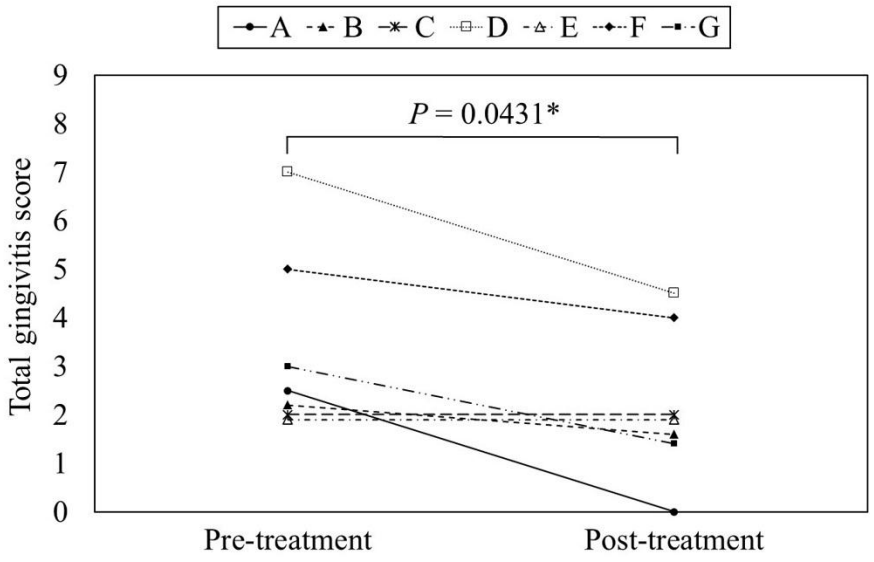
At the post-treatment period (i.e., one month after the start of treatment), physical and blood examination, observation of degrees of gingivitis, plaque/calculus deposits and halitosis and subgingival microbiota analysis were carried out in the same manner as the pre-treatment period. Significant differences between total scores at the two measurements were determined via Wilcoxon's signed rank-sum test. P values of <0.05 were considered significant. To perform statistical analysis, we used commercially available computer software (BellCurve for Excel®; Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results and Discussion

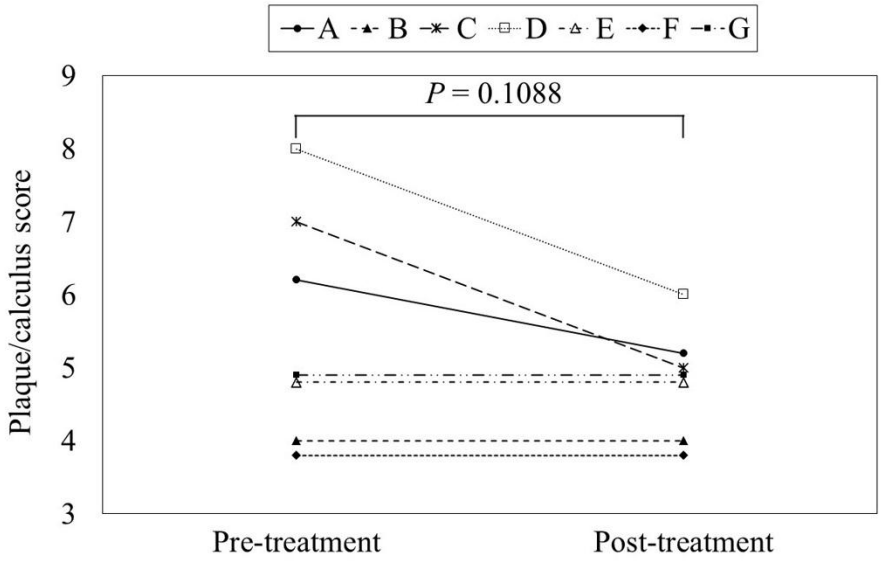
In this study, all the cats accepted the CNF gel readily during the administration period. The posttreatment physical and blood examinations revealed no adverse effects in any of the cats, and the CNF gel was therefore safe for use.

The gingivitis scores before and after treatment are compared in Fig. 2a. Before treatment, the total gingivitis scores of all cats ranged from 2 to 7 (median: 2.5), which were much lower than the maximum score of 12; thus, these cats had mild or moderate gingivitis. After treatment, the gingivitis scores in all cats decreased significantly (median: 2; range: 0–4.5; P = 0.0431). These findings may be elucidated by the fact that CNF can suppress the expression of inflammatory molecules, including nuclear factor κB, cyclooxygenase-2, and inducible nitric oxide synthase, which are involved in gingivitis (Lappin *et al*., 2000; Morton and Dongari-Bagtzoglou, 2001; Younis and Hassan, 2017). Hence, our data indicate that CNF treatment suppressed inflammation, thereby contributing to the improvement in feline gingivitis.

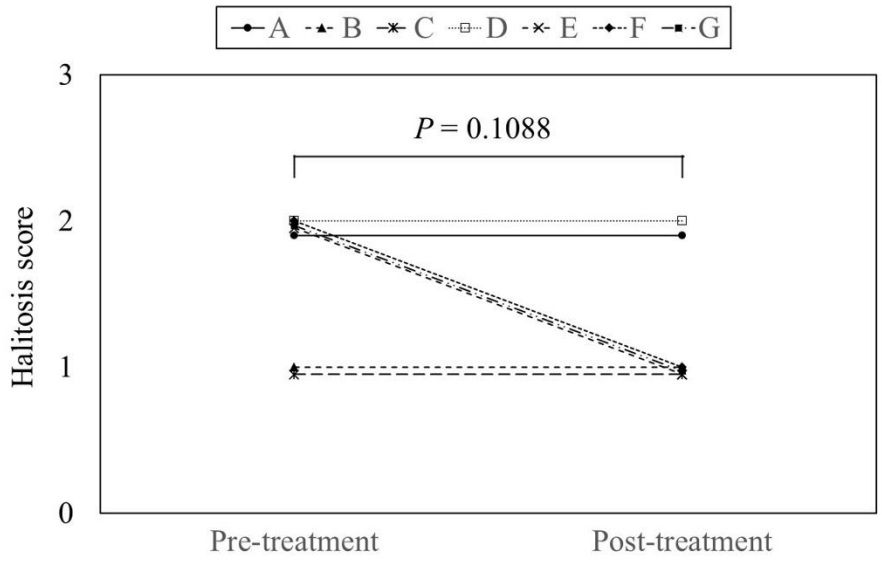
Chitin and its derivatives can inhibit bacterial growth by disrupting bacterial membranes (Je and Kim, 2006) and blocking DNA transcription in bacteria (Liu *et al*., 2000), and we therefore thought that it could suppress bacterial complications of feline gingivitis, including plaque/calculus and halitosis. Nevertheless, we found insignificant differences between pre-treatment scores and post-treatment scores of either, although both scores decreased after treatment (Fig. 2b and 2c). One of the reasons may be that in most of these cats, the scores of these manifestations were fairly low before treatment and so CNF had little effect. Further study of feline gingivitis with high plaque/calculus and halitosis scores is needed to elucidate the effect of CNF on these manifestations.



(a)



(b)



(c)

Figure 2 Temporal changes of scores of gingivitis (a), plaque/calculus (b), and halitosis (c) in seven cats (A–G) with gingivitis during the observation period. *P* values were calculated using Wilcoxon's signed rank-sum test.

We also evaluated subgingival microbiota in cats before and after treatment. Table 1 describes the relative abundance of the major five phyla identified by Rodrigues *et al*. (2019). The relative abundance of these phyla before and after treatment did not differ significantly, indicating that 1-month treatment with CNF has no significant impact on subgingival microbiota in cats. Of all the detected genera, the relative abundance of *Absconditabacteriales* (SR1), *Aquaspirillum*, and *Phaselicystis* significantly increased after treatment compared with before treatment (Table 1). In addition, the relative abundance of *Clostridia*_UCG-014 and *Peptostreptococcus* marginally decreased and that of *Saccharibacteria*, *Dojkabacteria*, and *Arcobacter* spp. marginally increased from before to after treatment. Pathogenic significance of these genera, except for *Peptostreptococcus* spp., in feline periodontal diseases was unclarified in the previous studies (Rodriques *et al*., 2019; Krumbeck *et al*., 2021), and thus needs further consideration in whether the change in the proportion of these genera can affect the prognosis of feline gingivitis. *Peptostreptococcus* spp. is

one of the more abundant bacteria in cats with periodontal disease than in clinically healthy cats (Rodriques *et al*., 2019; Krumbeck *et al*., 2021), implying that the decrease in its abundance might be related to the improvement of gingivitis score. To clarify the effect of CNF treatment on subgingival microbiota in cats, further studies would be needed.

Table 1 Relative abundance of major phyla of subgingival microbiota in seven cats before and after treatment

Name of phyla or genus	Relative abundance (%)[1)]		*P* value [2)]
	Pretreatment	Posttreatment	
Major phyla			
Proteobacteria	36.0 (22.5–44.7)	32.5 (10.2–39.0)	0.1763
Bacteroidetes	33.4 (17.8–38.2)	34.5 (14.9–38.7)	0.7353
Firmicutes	12.4 (8.3–17.5)	8.8 (7.3–14.9)	0.3980
Spirochaetes	4.7 (0.1–10.0)	3.6 (1.1–8.3)	0.7998
Fusobacteria	2.1 (0.8–6.0)	2.6 (0.8–6.1)	0.7353
Genus			
Absconditabacteriales (SR1)	0.835 (0.055–2.12)	1.510 (0.95–2.47)	0.0280*
Aquaspirillum	0.730 (0–2.46)	1.750 (0.04–5.09)	0.0425*
Phaselicystis	0 (0–0.045)	0.04 (0–0.05)	0.0431*
*Clostridia*_UCG-014	0.02 (0–0.41)	0 (0–0.145)	0.0679
Saccharibacteria	0.21 (0.07–0.29)	0.3 (0.05–0.78)	0.0630
Peptostreptococcus	2.23 (0.13–3.75)	1.58 (0.99–3.27)	0.0630
Dojkabacteria	0.04 (0–0.59)	0.165 (0–0.7)	0.0747
Arcobacter	0.355 (0–1.685)	1.95 (0–5.865)	0.0747

1) Median (range)
2) *P* values were calculated by Wilcoxon's signed rank-sum test.

There were several limitations in this study. Firstly, we used only a small number of cats without a control group, and studied with limited observation points and experiment period because animal experimentation takes animal welfare into consideration. Secondly, spontaneous gingivitis in all cats was relatively mild or moderate form, and thereby further trials for cats with the severe form of gingivitis should be considered. Finally, a pathological examination of the gingiva was not carried out at the pre- and post-treatment periods for a detailed assessment of the beneficial and adverse effects of CNF.

To our knowledge, this is the first study of the effects of CNF on feline gingivitis. We found slightly but significant improvement in gingivitis score in cats, some of which also showed improvement of plaque/calculus and halitosis scores after 1 month treatment with CNF gel. In addition, we confirmed the change of subgingival microbiota during treatment with CNF, although the clinical implications remain to be clarified. The present findings provide some support for the use of CNF as one of the treatment options for feline gingivitis.

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