Effect of passive immunization by anti-gingipain IgY on periodontal health of dogs

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Abstract

Anti-gingipain IgY (IgY-GP), known as hyperimmune γ-livetin from egg yolk, inhibits the enzyme activity, growth and adherence of *Porphyromonas gingivalis* to gingival epithelial cells. Our objective was to evaluate the efficacy of IgY-GP on periodontal health of dogs. IgY-GP was prepared from the egg yolk of hens immunized with the gingipain from *Porphyromonas gingivalis* ATCC 33277. Two *in vivo* trial models were conducted on 15 adult dogs with periodontitis by giving IgY-GP-supplemented dog feed for 8 weeks and direct application of the IgY in dental ointment to the periodontal pockets at weekly interval for 4 weeks. Clinical parameters including gingivitis, periodontitis, oral health index, bleeding on probe (BOP), pocket depth (PD), and dental calculus removal pattern for selected premolar teeth were recorded at baseline, 4 and 8 weeks post treatment.IgY-GP showed strong cross-reactivity with gingipain from *Porphyromonas gulae* and inhibited the enzyme activity *in vitro*. In the dog trials, IgY-GP resulted in significant improvement of oral health parameters including gingivitis and periodontitis scores, BOP, dental calculus removal. No adverse events during and after antibody applications were noted. Oral immunotherapy by using IgY-GP is a new promising alternative to conventional preventive and therapeutic methods to improve oral health status in dogs.

Introduction

Periodontitis is probably the single most common infectious disease in veterinary medicine especially in small animal practice.[1] The disease is caused by a group of black-pigmented anaerobic bacteria. Among them, *Porphyromonas gingivalis* has been considered to be a major periodontal pathogen because the bacterium is more frequently detected in active lesions of periodontitis in humans[2] and its subgingival implantation in mice,[3] rats[4] and non-human primates[5] is associated with initiation and progression of the disease.

Virulence of *P. gingivalis* is associated with the proteolytic enzymes gingipains[6] that are produced as secreted or membrane-associated forms by the bacterium.[7] Gingipains are cysteine proteinases that can degrade key components of the immune system.[8] In addition, gingipains are important for the bacterium to proliferate and survive in the periodontal pockets.[9] These facts suggest that gingipains are the most promising target for vaccination against periodontitis and related systemic diseases.

Immunotherapy by specific chicken antibodies (IgY) has been used with mixed successes against infectious diseases of viral, bacterial and fungal origin on both humans and animals.[10-12] Peroral administration with IgY is an attractive approach because IgY does not activate mammalian complement or interact with mammalian Fc receptors that could mediate inflammatory response in the gastrointestinal tract.[13] In recent papers we have reported that anti-gingipain IgY had preventive effect against periodontitis in human patients.[14] In the present study we examined if the same IgY has effect against periodontal diseases in companion animals when used in different applications.

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Materials and Methods

Bacterial strains and culture conditions

Porphyromonas gingivalis ATCC 33277, *Porphyromonas gulae* ATCC 51700, *Porphyromonas salivosa* ATCC 49407, *Porphyromonas circumdentaria* ATCC 51356 were obtained from the American Type Culture Collection. *Porphyromonas gingivalis* 381 and *Porphyromonas endodontalis* F2 and F5, 2 clinical isolates from the subgingival plaque of adult dog,[15] were kindly provided by University of Hokkaido (Department of Disease Control and Molecular Epidemiology, Health Sciences University of Hokkaido, Japan). All strains were maintained anaerobically on Brucella HK agar (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10% horse blood.

Preparation of gingipain and anti-gingipain antibody

P. gingivalis ATCC 33277 was used for production of gingipain (GP) and IgY-GP according to the methods described previously.[16] Partially purified IgY-GP is a polyclonal preparation and control IgY (prepared from non-immunized chicken eggs) samples were prepared from egg yolk by chloroform extraction and ammonium sulfate precipitation.[17] The antigen and antibody protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad laboratories, Berkeley, CA, USA). Enzyme linked immunosorbent assay (ELISA) was used to check the titer of the specific antibody IgY-GP as described previously.[16]

In vitro assays for IgY-GP

An indirect ELISA was used to determine the activity of IgY-GP against *P. gingivalis, P. gulae, P. salivosa, P. circumdentaria* and *P. endodontalis*. The bacteria were grown in enriched trypticase soy broth medium, harvested by centrifugation, washed and disrupted by sonication as described previously.[16] The obtained preparations were adjusted to 5 μg/mL concentration with 0.05 M carbonate buffer (pH 9.6) and used to coat ELISA plates (100 μL/well) at 4°C for 18 h. The indirect ELISA was performed as described previously. A similar procedure was used with purified gingipains from *P. gingivalis* as coating antigen. The highest dilution of IgY solutions showing an OD of more than 0.2 was used as the cut-off value for a positive reaction.

To examine the enzyme inhibition effect of IgY samples the following procedure was used. Gingipain preparations from various Porphy-

romonas spp. were activated in a buffer consisting of 200 mM HEPES, (pH 7.6), 5 mM $CaCl_2$ and 10 mM cysteine for 5 min at 37°C, mixed with IgY-GP or control IgY (50 mg/mL) in the same buffer, and incubated at 4°C for 1 h. The substrate N-α-Benzoyl-L-arginine-*p*-nitroanilide (BApNA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A 50 μL aliquot of this mixture was added to 150 μL of reaction solution consisting of 100 mM Tris-HCl buffer (pH 7.5), 5 mM dithiothreitol, 5 mM L-cysteine and 1 mM *N*-α-Benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA) (Sigma Chemical). The assay mixture was incubated at 37°C for 20 min and the reaction was stopped by adding 50 μL of 20% acetic acid. The release of p-nitroaniline was determined by measuring its absorbance at 405 nm.[16] The reaction without gingipains was used as the negative control to monitor background readings. One unit of gingipain activity was defined as the amount of enzyme releasing 1 μmol of p nitroanilide per minute in the reaction mixture under assay conditions and expressed as U/mL.

The cell damage assay was based on the protocol of Yokoyama *et al.*[16] In this assay, FaDu cells (Human pharyngeal carcinoma cell line; ATCC HTB-43, Manassas, VA, USA) were cultured overnight in minimum essential medium (Eagle's minimum essential medium, Nissui, Japan) containing 10% FBS in six-well microtiter plates. The plates were charged with serum-free medium containing gingipains (200, 100, 50 and 0 μg/mL), phosphate buffered saline (PBS) or gingipains from Porphyromonas spp pretreated with either IgY-GP or control IgY (50 mg/mL each) and incubated at 37°C for 1 h. The plates were washed 3 times with PBS to remove detached cells and the remaining cells were counted after trypan blue staining. A similar procedure was used on two other cell lines, KB cells (Human pharyngeal carcinoma cell line; ATCC CCL-17, USA.) and Ca 9-22 cells (Human gingival cell line; Japanese Cell Resource Bank, JCRB-0625, Shinjuku, Japan).

Effect of IgY-GP on dogs

All procedures that involved animals were approved by the animal care and use committee of animal research center, Kyodoken Institute, Kyoto, Japan. Mixed-breed dogs with different degrees of periodontitis were purchased from the commercial dog breeders. The dogs were housed individually in stainless steel cages in a temperature-controlled room (25°C) with a 12-hour light/dark cycle. Food and water were provided ad libitum. The dogs were physically healthy and had not been treated with any antimicrobials prior to the study. The dogs were placed under acclimation period for 7 days, during which they were fed a commercial diet (Aijou monogatari series, Yeaster Co., Hyogo, Japan.), a dry pellet food free of antimicrobials and probiotics.

Two experiments were conducted to examine the effect of IgY-GP on dogs. In the first experiment, 15 dogs were randomly divided into 3 groups (5 dogs per group): two test groups (test 1 and test 2, respectively) and one control group. The test 1 group (average body weight = 7.88±1.34 kgs and age = 74±13 months) was fed 35 mg IgY-GP per kg of body weight once a day. The test 2 group (average body weight = 8.04±1.49 kgs and age = 67±2 months) was fed 17.5 mg IgY-GP per kg of body weight twice a day. IgY-GP yolk powder was mixed with dry pellet food just prior to feeding and fed to the dogs for 8 weeks. The control group (average body weight = 8.38±1.91 kgs and age = 68±2.30 months) was fed the same dry feed supplemented with control IgY.

One premolar tooth was selected from each dog for examination at baseline (one day before treatment), 4 and 8 weeks post treatment. Neither scaling nor root planning (SRP) was done supra-and subgingivally for these experimental teeth. The examiner was not involved in the treatment process and was not aware of the dog group assignment. The examination parameters included gingivitis and periodontitis scores, bleeding on probe (BOP), pocket depth (PD) and dental calculus removal status. During the oral examination, the dogs were sedated by intramuscular injection of medetomidine hydrochloride (0.05 mg/kg) (Medetomidine hydrochloride; Domitor; Meiji Seika Kaisha, Tokyo, Japan) and then anti-sedated with atipamezole hydrochloride (0.05 mg/kg) (Atipamezole hydrochloride; Antisedan; Meiji Seika Kaisha). Photographs were taken at the time of oral examination. The gingivitis and periodontitis levels were scored as follow: score 0 = normal; 1 = mild; 2 = moderate; and 3 = severe inflammation. The oral health index was calculated as the sum of scores with 0 point indicating optimal periodontal health and 6 points or higher indicating poorest periodontal health.[18] BOP was assessed by the presence or absence of bleeding 30 sec after probing.[14,19] PD was measured by the use of a standard periodontal probe. For evaluation of dental calculus removal status, one premolar tooth per dog was selected. dental calculus thickness and areas were assessed through the comparison of photographs taken for representative teeth by three independent veterinarians. Mean values are expressed as dental calculus removal percentages.

In the second experiment, five dogs (average body weight = 6.6±1.98 kgs and age = 70±2 months) were used. Two pairs of contralateral premolar teeth per each dog (4 sites/dog) were selected to check the effect of IgY-GP by a split mouth design.[14,19] The selected teeth all had clear gingival inflammation with a PD ≥5 mm. One tooth in each contralateral pair was treated with 20% IgY-GP mixed in dental ointment (a neutral gel containing hydrocarbon gel, sucrose esters of fatty acids and hydroxypropylmethylcellulose 2208) (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) (test sites, n=10), whereas the other tooth was treated with dental ointment only (control sites, n=10). The dental ointment was administered 4 times (200 mg /pocket/time) at weekly interval into the periodontal pocket by a root canal syringe without pretreatment by scaling and root planning (SRP). Clinical parameters were recorded and scored for at baseline, 1, 2, 3 and 4 weeks post treatment as described in the first experiment. PD was measured at baseline, 2 weeks and 4 weeks post treatment.

All data are presented as the means±standard deviations (SD). The statistical significance was evaluated by Chi-square, ANOVA and Student's *t*-test where appropriate. A value of $P<0.05$ was considered to be statistically significant.

Results

In vitro efficacy of IgY-GP

The activity of IgY samples was measured by ELISA. The titer of IgY-GP was 128,000 while that of the control IgY was less than 100. The reactivity of the IgY-GP with different Porphyromonas spp. is shown in the Table 1. IgY-GP strongly reacted with *P. gingivalis* and *P. gulae* but showed weak cross activity with *P. salivosa.* IgY-GP did not cross-react with *P. cir-*

Table 1. Cross-reactivity of IgY-GP with different *Porphyromonas* species.

Porphyromonas species	IgY-GP	Control IgY
P. gingivalis ATCC 33277	1.276±0.01°	0.015±0.03
P. gingivalis 381	1.196±0.01	0.000±0.02
P. gulae ATCC 51700	0.833±0.02	0.019±0.01
P. salivosa ATCC 49407	0.227±0.04	0.010±0.01
P. circumdentaria ATCC 51356	0.064±0.01	0.040±0.02
P. endodontalis F2	0.094±0.01	0.010±0.004
P. endodontalis F5	0.057±0.02	0.015±0.002

Absorbance at 492 nm; °the highest dilution of IgY-GP showing an OD of more than 0.2 was used as the cut-off value for a positive reaction; [b]mean OD±standard deviation. Data shown are the means of three independent experiments.

cumdentaria and clinical isolates of *P. endodontalis* F2 and F5. Control IgY showed no reaction with all Porphyromonas spp.

Very high proteolytic activity was detected in gingipain preparations isolated from the two *P. gingivalis* strains and *P. gulae* ATCC 51700 strain. Weak proteolytic activity was observed in the preparation from *P. salivosa* ATCC 49407 strain and no such activity was found in the preparations from *P. circumdentaria* ATCC 51356 and the 2 *P. endodontalis* strains. In the enzyme inhibition assay, IgY-GP inhibited the proteolytic activity of gingipain preparations (200 μg/mL) from *P. gulae* strain ATCC 51700 and *P. gingivalis* strain 381 by 50% (Figure 1) and 60%, respectively.

In the cell damage assays, gingipains from *P. gulae* added to FADU monolayer cell culture resulted in cell death and subsequent detachment from the plates (Figure 2A) in a dose-dependent manner (Figure 2B). Pretreatment of gingipains with IgY-GP protected the cells from damage and significantly increased cell survival compared to the non-treated control groups (P<0.05, one-way ANOVA) whereas that with control IgY did not show any protection effects (Figure 2). Similar results of cell protection were obtained when the 2 other cell lines (KB and Ca9-22) were used in separate experiments (*data not shown*).

Effect of IgY-GP on dog

All the dogs used in the experiments remained healthy throughout the test period. No allergy reaction or any side effects were observed on any dog administered with the test and placebo samples. The effect of IgY-GP on dog oral health status is shown in Table 2 and Table 3 for the first and second experiment, respectively. In the first experiment, the mean scores for all examined parameters including gingivitis, periodontitis, oral health index, and bleeding on probe, were significantly lower after 8 weeks compared to the baseline (P<0.05 or P<0.01 depending on parameters, one-way ANOVA) (Table 2). There were no significant changes in any parameters in the control group at 4 and 8 weeks. No remarkable differences were observed for all parameters between test 1 and test 2 groups. The effect of IgY-GP on oral health was also clearly seen in experiment 2 where most examined parameters were significantly lower starting from 2nd week post treatment. No significant changes were noticed for any parameter in the control group (Table 3).

The changes in pocket depth (PD) for both experiments are shown in the Figure 3. In the first experiment, only the test 1 group had lower PD at 4 week and 8 week but the changes were not significant (P>0.05; Student's *t*-test, Figure 3A). In the second experiment, the test group demonstrated a significant reduction (P<0.05, Student's *t*-test) in PD at 8 weeks post treatment. The mean PD in this group was 6.6 mm±2.4, 4.9 mm±2.15, and 4.2 mm±2.5 at baseline, 2 weeks and 4 weeks post treatment, respectively. There were no significant changes in PD in the placebo group (Figure 3B). There was also significant difference in the mean PD between both groups at 8 weeks

Table 2. Effect of IgY-GP supplemented with dry food on oral health parameters of dogs with periodontitis.

Parameters	Test 1° (n=5)			Test 2# (n=5)			Control (n=5)		
	Baseline	4 wk	8 wk	Baseline	4 wk	8 wk	Baseline	4 wk	8 wk
Gingivitis§	1.2±0.8	0.7±0.48	0.2±0.42*	1.2±0.42	0.7±0.48**	0.8±0.42**	1.7±0.95	1.6±0.84	1.5±0.97
Periodontitis^	1.1±1.1	0.1± 0.32*	0.0± 0.0*	1.2±1	0.2±0.42*	0.2±0.42*	1.4±1.1	0.9± 1.2	0.8± 1.0
Oral health index§	2.3±0.07	0.8 ± 0.4	0.2±0.1	2.4± 0	0.9±0.35	1.0±0.42	3.1±0.2	2.5±0.5	2.3±0.5
Bleeding on probe°°	4/5	1/5	1/5	5/5	0/5	0/5*	5/5	5/ 5	5/5

°The test 1 group was fed 35 mg IgY-GP per kg of body weight once a day; #the test 2 group was fed 17.5 mg IgY-GP per kg of body weight twice a day; §,^,§gingivitis and periodontitis levels were evaluated visually and scored as follow: score 0 = normal; 1 = mild; 2 = moderate and 3 = severe inflammation. The oral health index was calculated as the sum of gingivitis and periodontitis scores with 0 point indicating optimal periodontal health and 6 points or higher indicating poorest periodontal health. °°Bleeding on probe was assessed by the presence or absence of bleeding 30 sec after probing. Values reported are no. of sites with bleeding/no. of sites examined. *P<0.01, **P<0.05 compared to baseline.

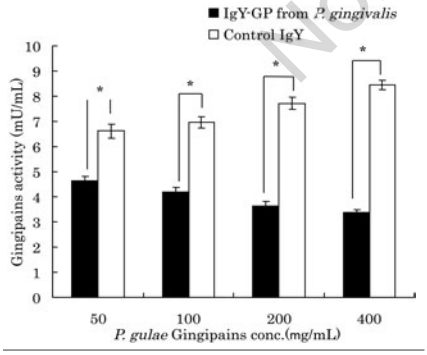

Figure 1. Effect of IgY-GP on the cross protease activities of gingipain extracted from *P. gulae*. The inhibitory effects of IgY-GP from *P. gingivalis* and control IgY (50 mg/mL each) on gingipain activity were evaluated. Data are shown as the means± SD of three independent experiments. *P<0.01 indicates a significant difference among study groups according to Student's *t*-test.

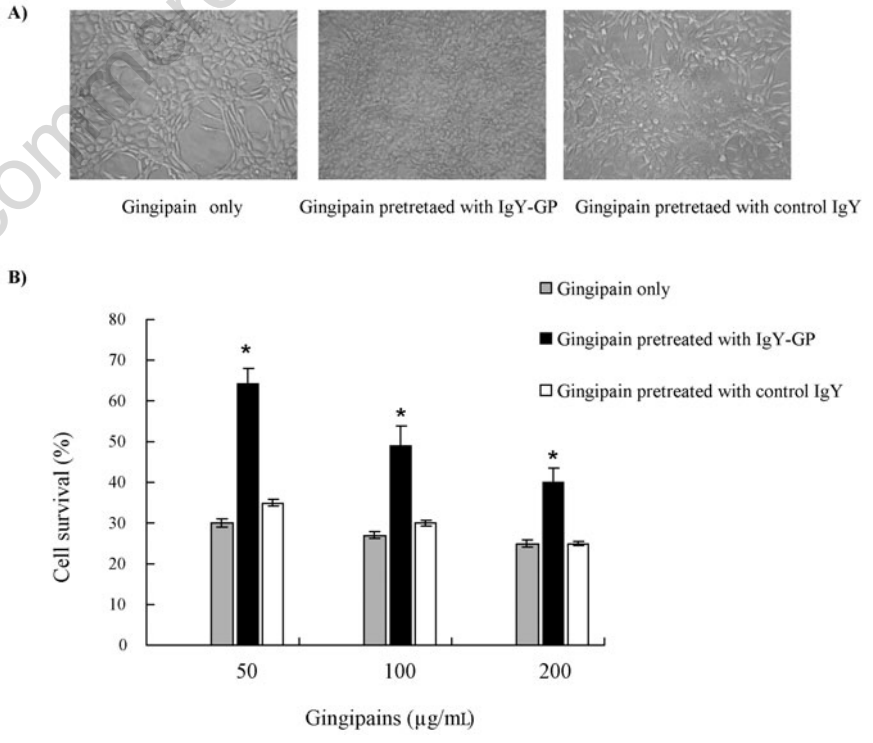

Figure 2. Effect of IgY-GP on the epithelial cell attachement. A) Microphotographs of the morphological changes of FaDu cells treated with gingipains extracted from *P. gulae* and pretreated with IgY-GP or control IgY (×200 magnification). B) Inhibition of gingipain-induced cell damage by IgY-GP or control IgY. Data are shown as the means±SD of three independent experiments. *P<0.01 (Student's *t*-test) indicates significant differences between the IgY-GP compared to the control IgY groups.

(P<0.05, Student's t test). The dental calculus removal pattern results in the first experiment are shown in the Table 4. At 4 weeks post treatment, 3 and 1 dog in the test 1 and test 2 groups, respectively, demonstrated dental calculus removal with the reduction of tooth surface areas of 26% and 4%, respectively. The figures for the 2 groups at 8 weeks were 4 and 2 dogs, respectively, and 42% and 22% reduction in dental calculus area, respectively. No dental calculus removal was seen in the control group. There was a significant difference in the number of dogs with dental calculus removal between the test 1 and control group at 8 weeks post treatment (P<0.05, χ^2). Figure 4 shows the change in dental calculus removal pattern in 3 dogs in the test groups. At 8 weeks these dogs show clear reduction of dental calculus build-up on the teeth.

Discussion

This study was undertaken to investigate the *in vitro* and *in vivo* efficacy of egg yolk antibody against *P. gingivalis* gingipains (IgY-GP) in prevention and treatment of gum diseases in dogs. The most significant finding in this study is the fact that IgY-GP in various experiment settings resulted in significant improvement of oral health status in the dogs.

Black-pigmented bacteria have been known to cause periodontitis and gum diseases in human and animals. Several bacterial species have been reported to be the causative agents of periodontitis in dogs which include *P. gingivalis*, *P. gulae*, and others.[20] Although gingipain is known to be an important virulent factor of these bacteria, no studies to compare the enzyme from different black-pigmented bacteria have been conducted to date. In the present study we demonstrated for the first time that IgY against gingipain from *P. gingivalis* cross-reacts with the same enzyme expressed by *P. gulae* and protects various cell lines from damages by the enzymes expressed by both *P. gingivalis* and *P. gulae*. The IgY did not react with *P. circumdentaria* and *P. endodontalis* isolates because these bacteria are non-protease bearers. The role of these bacteria in periodontitis development is uncertain and need to be investigated.

To investigate the efficacy of IgY-GP in dogs we designed 2 sets of experiments. In the first experiment we checked the preventive effect of IgY-GP on the dogs by mixing the IgY samples directly with dry pellet food. We choose 2 different feeding regimes based on an assumption that IgY concentration and retention in the mouth cavity could potentially affect the outcome. In both feeding regimes IgY-GP showed comparable effect on oral health status which is demonstrated by significant improvement of important parameters such as gingivitis, periodontitis, BOP, and oral health index. These results indicate that IgY-GP is useful in reduction of inflammation in oral cavity and prevention of periodontitis and gum diseases in dogs. The effect of IgY-GP on dental calculus removal pattern was exciting because dental calculus on teeth can be removed only by mechanical methods. Although the mechanism behind this effect is still unclear, specific IgY against gingipain may weaken the biofilm formed on teeth surface and result in the change in structure of dental calculus. The better dental calculus removal pattern in the test 1 compared to the test 2 group suggests that higher concentration of IgY-GP in oral cavity may be necessary to prevent tartar build up on teeth surface.

Although feeding IgY-GP with dry feed resulted in reduction of inflammation in dog oral cavity, the antibody did not have an effect on PD after 8 weeks of continuous feeding (Figure 3A). A possible explanation for this lack of effect is the IgY may not go deeply into periodontal pockets. In the second experiment, we applied IgY-GP in the form of ointment directly into some periodontal pockets 4 times at weekly interval. In this experiment not only inflammation-related parameters were significantly improved (Table 3), but the PD was also significantly reduced at 4 weeks after treatment (Figure 3B). The results suggest that IgY-GP may be useful for treatment of periodontitis. The effects of IgY-GP on various inflammation parameters in the dog oral cavity can be explained by its inhibition on gingipain expressed and released by pathogenic black-pigmented bacteria. IgY-GP is also known to inhibit growth of *P. gingivalis* in *in vitro* experiments.[21]

Various feed additives and systemic or local antimicrobial immunotherapeutic agents have been used so far for the treatment of periodontitis in companion animals. To our knowledge,

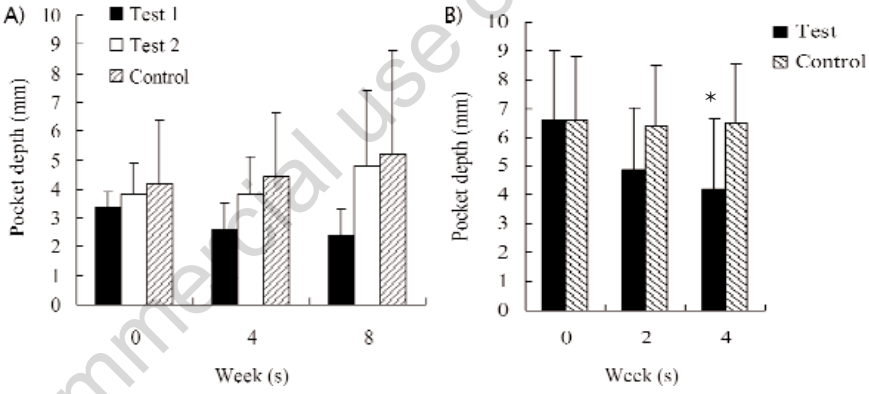

Figure 3. Effect of IgY-GP on the changes in probing depth in dogs when supplemented with A) dry food in experiment 1 and with B) dental ointment in experiment 2. Results are means±SD; *P<0.05 indicates a significant difference between the test and control groups.

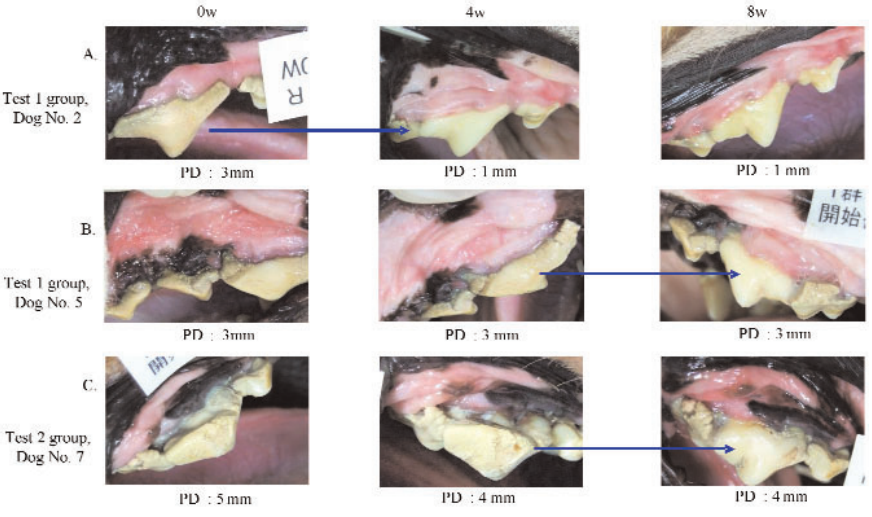

Figure 4. Representative dental photographs of 3 experimental dogs (A, B and C) showing the effect of IgY-GP on dental calculus removal. The same tooth was photographed before, 4 weeks and 8 weeks after feeding with the IgY-GP. PD, pocket depth.

Table 3. Effect of IgY-GP supplemented with dental ointment on oral health parameters of dogs with periodontitis.

Parameters	Test (n=10 sites)					Control (n=10 sites)				
	Baseline	1 wk	2 wk	3 wk	4 wk	Baseline	1 wk	2 wk	3 wk	4 wk
Gingivitis°	2.3±0.67	1.6±0.7	0.9± 0.57*	1.1± 0.74*	1.0± 0.82*	2.2±0.62	1.8±0.63	1.4±0.52	1.2±0.42	1.9±0.74
Periodontitis§	1.9±0.57	1.7±0.820.9±0.74**		1.1±0.88	0.7±0.82*	2.2±0.63	2.1± 0.74	1.5±0.70	1.3± 0.82	2.1± 0.74
Oral health index^	4.2±0.28	3.3±0.07	1.8± 0.00	2.2± 0.00	1.7± 0.20	4.4± 0.00	3.9± 0.20	2.9±0.07	2.5± 0.07	4.0±0.14
Bleeding on probe§	10/10	10/10	1/10*	1/10*	0/10*	10/ 10	10/10	10/10	10/10	10/10

°,§,^Gingivitis and periodontitis levels were evaluated visually and scored as follow: score 0 = normal; 1 = mild; 2 = moderate and 3 = severe inflammation. The oral health index was calculated as the sum of gingivitis and periodontitis scores with 0 point indicating optimal periodontal health and 6 points or higher indicating poorest periodontal health. §Bleeding on probe was assessed by the presence or absence of bleeding 30 sec after probing. Values reported are no. of sites with bleeding/no. of sites examined. *P<0.01, **P<0.05 compared to baseline.

Table 4. Dental calculus removal pattern in dogs fed with IgY-GP (Test 1 and 2) or control IgY-supplemented feed.

Group	No. of dogs showing dental calculus removal pattern (%) on tooth surfaces	
	4 weeks	8 weeks
Test 1° (n=5)	3 (26%)	4* (42%)
Test 2# (n=5)	1 (4%)	2 (22%)
Control	0 (0%)	0 (0%)

°The test 1 group was fed 35 mg IgY-GP per kg of body weight once a day; #the test 2 group was fed 17.5 mg IgY-GP per kg of body weight twice a day. *P<0.05 indicates a significant difference between test 1 and control group.

this is the first report on the use of IgY-GP for prevention and treatment of periodontitis in dogs. Compared to antimicrobial drugs IgY from chickens has various advantages due to its specific activity and its localized and non-invasive nature, which make IgY very safe especially when used for a long period of time.[13,22] In our study we also did not see any side effects in dogs during 8 weeks of continuous administration of the IgY. This fact together with the effects on various inflammation-related parameters indicates that IgY-GP is a valuable tool for prevention and adjunctive treatment of periodontitis in dogs.

Conclusion

It is concluded that oral immunotherapy by using IgY-GP is a new promising alternative to conventional preventive and therapeutic methods to improve oral health status in dogs.

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