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Effect of IgY on Periodontitis and Halitosis Induced by *Fusobacterium nucleatum*

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Running title: IgY prevents halitosis and periodontitis

Abstract

Fusobacterium nucleatum is a morbific agent in periodontitis and halitosis. Egg yolk antibody (IgY) was obtained from egg yolks afterward *F. nucleatum* stimulated chicken. This study was to access the effectiveness of IgY on periodontitis and halitosis lured by *F. nucleatum in vitro* and *in vivo*. The growth of *F. nucleatum* was inhibited ($p<0.05$) by different concentration IgY *in vitro* and the results of Halimeter shows volatile sulfur compounds (VSCs) were reduced to 904±57 ppb at concentration 40 mg/ml of IgY. The changes of fatty acids of *F. nucleatum* were determined using GC-MS. The scores for odor index of rat saliva were decreased. The major constituent of volatile organic compounds (VOCs) including short chain acids decrease 46.2% in 10mg/ml IgY, ammonia decrease 70% in 40mg/ml IgY, aldehydes and olefine ketones are almost unchanged. The ELISA assay revealed that IL-6 and TNF-α were decreased after 4 weeks' IgY treatment. Morphometric (X-Ray) and histological analyzes (HE) shows that IgY reduced alveolar bone loss and collagen fibers became orderly in rat models. As a result, IgY may have the potential to treat periodontitis and halitosis.

Keywords

Egg Yolk antibody; *Fusobacterium nucleatum*; halitosis; inflammation; alveolar bone loss

Introduction

Halitosis, a nasty odor emanating from the mouth, is a multi-factorial disease. Bad breath primarily is connected with volatile sulfur compounds (VSCs) including hydrogen sulfide (H_2S) and methyl mercaptan that produced by bacteria [1]. Moreover, butyric acid, methylamine, indole, dimethylamine, cadaverine, acetone and propionic acid are named as volatile organic compounds(VOCs), which may contribute oral malodor [2,3]. The main cause of oral malodor is associated with tongue coating, gingival and periodontal disease.

Periodontitis is a chronic inflammatory disease of multifactorial etiology which is mainly caused by pathogenic bacteria. Periodontal disease can lead to pain, uncomfortable, impaired tissues of the teeth, and tooth loss [4]. The infection will spread and affect the entire periodontium, even cause cardiovascular disease [5]. While oral malodor is not directly caused by periodontal disease, abundant evidences indicate that periodontal disease intensified the severity of malodor. According to the reports, approximately 60% patients with periodontitis produce volatile sulfur compounds (VSCs) [6, 7].

Among oral bacterium, *F. nucleatum* that is a Gram-negative anaerobe has pathogenic potential and is involved in halitosis as well as periodontal diseases [8]. *F. nucleatum* is in a minority in the oral cavity, which is widely detected elsewhere in the body of infectious conditions [9]. *F. nucleatum* has a strong invasion activity to human gingival epithelial cells, which directly contribute periodontal disease or by forming a bridge with other virulent anaerobes [10]. Meanwhile, *F. nucleatum* produces large amounts of volatile sulfur compounds (VSCs) and is a representative bacterium for halitosis [1]. Available treatments, such as chemical antiseptics, antibiotics and mechanical therapy, are effectively used for halitosis and periodontitis. However, the antibacterial-resistant bacteria and by-effect of antibiotics limit antibacterial therapy [11]. Once treatments are discontinued, simultaneous phenomenon will become clearly apparent. *F. nucleatum* produces malodor during periodontitis development in gum. To treat halitosis, the main strategy would reduce the components of oral malodor that produced by microorganisms in the oral cavity.

Recently, chicken egg yolk antibody (IgY) that obtained from egg yolks after immunizing chicken by distinctive antigens, has attracted much attention as a reliable and cost-effective immunization method to alleviate many diseases [12]. It was proved that IgY was applied against caries caused by bacteria in rats [13], and then in human in addition [14]. IgY can

inhibit bacterial enzyme activity, neutralize toxin and decrease cell adhesion of microorganisms to reduce microbial infections. Anti-*P. gingivalis* egg yolk antibody, an immunotherapeutic agent, was verified to be powerful in the medication of periodontitis. Hamajima had demonstrated that IgY suppresses the increasing of the biofilm on the teeth surface by binding a 40-kDa out membrane protein of *Porphyromonas. gingivalis* [15]. IgY inhibits the growth of *Solobacterium moorei* that is a pathogenic agent in disease like bad breath, periodontitis and other infections [16]. Xu et al. had investigated that IgY has a positively effective inhibition on *F. nucleatum* [17]. However, there are no studies that using IgY to remedy periodontal inflammation affiliated with halitosis caused by *F. nucleatum*. Therefore, this study discussed that IgY was extracted from egg yolks after *F. nucleatum* stimulates chicken have a positive treatment of halitosis and periodontitis caused by *F. nucleatum*.

Materials and methods

Bacterial strain and culture conditions

F. nucleatum (ATCC 25586) used in the present study was brought from American Type Culture Collection (Manassas, VA, USA). The bacteria amplified in brain-heart infusion broth (Hopebio, Qingdao, China) adding 0.5 mg/mL of yeast extract (Hopebio, Qingdao, China), 1 μg/mL of menadione (Adamas, Shanghai, China) and 5 μg/mL of hemin (Adamas, Shanghai, China). Modified artificial saliva (AS) was prepared following the instructions of Pratten et al. and Wong L et al. [18]. AS was used to culture F. nucleatum in anaerobic environment. *F. nucleatum* was cultured at 37 ℃ under anaerobic condition by using anaerobic pack (MITSUBISH, Japan) without shaking.

The growth inhibition of *F. nucleatum*

The growth inhibition assay was performed according to the previous method [19]. After amplifying in BHI media, the *F. nucleatum* was harvested by centrifugation and adjust to a final concertation at an OD 650nm of 0.5 (10^9 CFU/ml) in the AS. IgY against *F. nucleatum* (extracted from egg yolks after chickens were challenged with *F. nucleatum*) obtained from Maxam Ltd. (Shanghai, China). IgY were reconstituted to 40 (high-dose), 20 (mid-dose), 10 (low group) mg/ml in 3.9 ml liquid medium. And preparation AS that is free of IgY were used as negative and blank control. 200ul *F. nucleatum* liquid added 1.8ml AS and bacterial solutions were incubated at 37℃ in the anaerobic environment. Aliquots (2ml) were taken at 0, 2, 4, and 6h of bacterial to sterile tubes, and absorbance was measured at 650nm using a spectrophotometer (V5100B, METASH Ltd., Shanghai, China).

VSCs measurement with Halimeter

The bacteria were cultured in a 2ml glass bottle containing AS media with different concentration IgY. The air above medium in the bottle was detected. The changes of VSCs were assessed using the Halimeter (Interscan Corp., CA, USA) that is a sulfide monitor. *F. nucleatum* mingled different concentration IgY artificial saliva was incubated at 37 °C for 48h using anaerobic pack (MITSUBISH, Japan) without shaking. Then a new 0.25 diameter plastic straw was inserted into the air inlet of the Halimeter and another end of the plastic straw was attached to the test tubes headspace to measure levels of VSC. The three parallel maximum readings in parts per billion (ppb) three parallel readings values were recorded [20].

Measurement of fatty acids in media

In order to detect fatty acids, the artificial saliva needs to be concentrated. The media were filtered through poly-sery HLB solid phase extraction(SPE) column (CNWBOND HC-C18, Anpel, China), and washed thoroughly with 1ml methanol (CNW HPLC-grade, Anple,

China). Then the liquid was transferred to 1.5 ml-tube for analysis of fatty acids. Aglilent 7890A was used as a chromatography solution system (Agilent, USA) supplied with a HP-5 MS capillary column (30 m × 250 μm× 0.25μm; crosslinked (5% -phenyl) -methylpolysiloxane; Agilent Scientific, Folsom, CA). Sample (2 μl) was injected by the 25ul syringe (Shanghai Guangzheng medical instrument company, Shanghai) A split ratio was set at 1:1. The carrier gas used helium setting a constant flow rate of 1 mL/min. The inlet temperature set at 250 °C. The GC column temperature rose to 285 °C at a rate of 8°C/min and then maintained at 285 °C for 5 min. AMSD 5973 mass-spectrometer choose ion monitoring mode and focused at m/z 40-400 molecular ion. The temperature of the source is 230 °C. Mass spectra were obtained at an energy level of 70 eV. The structure of sample was identified by National Institute of Standards and Technology (NIST) library. According to the software provided by the instrument, the interference peak is removed, then chemical is integrated. The proportion different components relative contention (Ab*s) is calculated.

Establishment of periodontitis rat model

Specific-pathogen-free female Sprague Dawley (SD) rats (n=10, weight 150g±10g) were purchased form SLA Laboratory Animal Co., Ltd (Shanghai, China). Rats were fed in individually cages and afforded sterile food and water under specific-pathogen-free condition. Rats were used for experiments when they are 9-10 weeks old. The Ethics Committee of Shanghai Ocean University (Shanghai, China) agreed that animals experience and study protocol. For establishment of a halitosis with periodontitis rat model, maxillary bilateral second molar of the rats were ligatured with a sterile 5-0 silk (Sangon Biotech Co., Ltd., Shanghai, China). Then 100μL live *F. nucleatum* (10^8 CFU/ml) mixed in PBS was smeared into every rat oral cavity three times a week and lasting four weeks to induce periodontal infection. After successful modeling, the diseased rats were divided into three groups (n = 3)

randomly and gave different treatments. In the experimental groups, 200 µl different concertation IgY solutions were smeared one times into the oral cavity one day for 30 days. The same volume of 0.9% NaCl solution served as a negative control.

Organoleptic Measurements

Two experienced odor judges accessed malodor levels, as previously reported [21]. Judge scores were recorded on a range of 0 to 5, as follows: score 0, no distinct odor; score 1, almost no noticeable malodor; score 2, minor, but clearly notable malodor; score 3, mild malodor; score 4, strong malodor; score 5, extremely intensive malodor. The saliva was obtained by sterile cotton swabs wiping 30 seconds in the different treatment rats' oral cavity. Swab was putted immediately into a 10 ml headspace vial with a screw top lid (CNW, Anple, Shanghai, China). Judges immediately sniffed the malodor emanating from each test tube to access test tube malodor levels afterward swashing and unscrewing the test tubes.

Measurement of malodour compounds from rat saliva

The new collection of saliva putted into a vial and placed in a water bath adjusted to 45 °C. Three replicates per each sample were prepared and analyzed. After 30 minutes, headspace VOCs were volatilized and adsorbed on a SPME fiber (65µm, PDMS/DVB; Anpel, Shanghai, China). SPME fiber were desorbed at 250 °C for 5 min in the injector port of a GC connected with a mass detector with a scan range from 40 to 400. The initial column temperature was set at 50 °C, then ascended at 10 °C/min to 150 °C then 5 °C/min to 250 °C and maintain for 2 min. The conditions of MS follow the above.

IgY effect on levels of IL-6 and TNF-α

159 Heart blood was collected from the groups after 4 weeks' different treatment. After
160 centrifugation at 12000 rpm for 8 min, the supernatants were transferred to a new tube. On the
161 basic of the manufacturer's instructions, using enzyme-linked immunosorbent assay kits
162 (ELISA) (Mlbio, Shanghai, China) to measure the concentration of TNF-α and IL-6. All
163 samples were measured in triple repetition with provided standards. The ELISA wells were
164 examined using the Microplate reader (SH-100, USA) at a wavelength of 450 nm.

165 **Histopathological Analysis**

166 After collecting the blood sample, the maxillary molar regions of rats were resected from
167 each rat and were fixed in 4% paraformaldehyde sodium phosphate buffer for 36 hours.
168 Radiographic images of the maxillary were taken by an X-ray machine (Faxitron MX-20
169 DC12 system; 119 Faxitron Bioptics, Lincolnshire, IL, USA). The bilateral mandibles were
170 demineralized in formic acid (10%) at room temperature for one week. Then bilateral
171 mandibles were embedded in paraffin; bilateral mandibles were cut into 4 μm thick using a
172 microtome (Leica RM2235, Germany). Samples were stained with hematoxylin and eosin
173 according H&E protocol using light microscopy (Lecia, Germany) to observe histopathology.

174 **Statistical analysis**

175 Statistical analysis was performed using SPSS 18.0 ((SPSS Inc., Chicago, IL, USA). All
176 data were represented as means ± standard deviation (S.D.) and were analyzed using
177 Student's t-tests. $P < 0.05$ was considered statistically significant.

178 **Results**

179 **Growth inhibition of IgY to *F. nucleatum* *in vitro***

To examine the capability of IgY to inhibit growth of *F. nucleatum* in artificial saliva, we cultured bacteria in the tubes adding different concentration IgY for 0, 2, 4 and 6h. Growth curves of *F. nucleatum* in media with IgY concentrations of high-dose (40mg/ml), mid-dose (20mg/ml), low-dose(10mg/ml) and blank are shown in Figure 1. After adding IgY, the turbidity of media decreased. The phenomenon mainly stems from the fact that *F. nucleatum* increased after the treatment with IgY. In these cases, growth was significantly inhibited by the addition of high dose specific IgY (p<0.01) compared with the control group. 10mg/ml (low-dose) and 40mg/ml (mid-dose) groups had less of a decrease in bacterial counts than high-dose one. The capability of 10mg/ml (low-dose) group to inhibit growth is similar to 20mg/ml (mid-dose) (p<0.05) compared with the control group.

The inhibition of VSCs by IgY

Halimeter is an effective instrument that measures total breath VSCs by electrochemical sensors. VSCs production by *F. nucleatum* was measured in the process of different dose IgY. As shown in Table 1, the concentration of IgY was connected with the production of VSCs. More specific, high-dose IgY was associated with the production of 904±57 ppb compared to 1921±99 ppb in the absence of IgY. In other words, there is a significant trend of decreasing after adding the high-dose IgY (p<0.01). Supplementing the culture medium with mid-dose IgY and low-dose IgY only slightly decrease VSCs levels produced by *F. nucleatum*. The inhibition effect increased as the concentration of specific IgY increased in the range of medium to high concentrations. The effect of mid-dose IgY on inhibiting VSCs is almost the same as that of low concentration.

The changes of fatty acids *in vitro* by IgY

Fatty acids produced by microbial metabolism are significant characteristics in species and quantities. Most bacterial acids are bounded with arguer molecules, such as phospholipids, glycolipids. Free acids, which are extracted with solvents, are found by GC-MS. The long chain fatty acids were detected by GC-MS. C16:0, C 16:1 and C18:0, the main component from the metabolic production of *F. nucleatum* in the artificial saliva, accounted for about 80% in fatty acids. No marked differences were found between control group and IgY groups about C18:1. The results indicate that the relative content of C16:1 decrease 28.6% compared with the control in Fig. 2. Trace amounts of 14-methyl-C15:0 were detected in artificial saliva. The percentage of C18:0 and C16:0 significantly decreased ($p<0.05$) after the treatment of high-dose IgY.

The changes of odor index

When *F. nucleatum* was incubated in mice oral, oder judge scores level is high. Furthermore, addition of high-dose IgY (40 mg/ml) to the rat oral significantly lowered both judge scores (Figure 3) ($p<0.05$), as compared with blank control. In the presence of 10 mg/ml IgY, malodor production was higher than high-dose IgY, but judge scores were lower than blank control ($p<0.05$).

The changes of volatile organic compounds (VOCs) *in vivo* by IgY

The Halimeter does not record all the odor vectors present in the breathed air. Volatile organic compounds (VOCs) in saliva of animal models were detected using SPME-GC-MS. A total of 17 compounds were founded to be ubiquitous in all samples in the present study, which included 4 alcohols, 4 carboxylic acids, 3 ketones, 2 aldehydes, 2 esters and 1 nitrogen compound (Table 2). According to the previous literature, all the compounds were divided into six categories. A1 represents alcohol, A2 represents aldehydes, A3 represents ketone, A4

225 represents carboxylic acid, A5 represents ester, C represents nitrogen compounds. These
226 VOCs are dominant classes of organic volatiles represented in the microbial production and
227 release of chemicals. The proportion of different components relative contention (Ab*s)
228 changes as shown in Figure 4. Short chain acids account for the largest proportion, with
229 butyric acid accounting for about 13% of short chain acids. After treatment of IgY, butyric
230 acid, hexanoic acid, propionic acid and acetic acid are significant reduced (p<0.05). Another
231 important substance, ammonia (C), is decreased by high-dose (40mg/ml) and low-dose (10
232 mg/ml) IgY. The high decrements of abundances contain short chain acids, ammonia and
233 alcohol, which indicates that these ingredients are sensitive in volatile odors. High-dose IgY
234 significantly decrease ingredients that have pungent smell, low-dose IgY decreased the
235 amount of reduction is less than high-dose IgY (Figure 4). However, aldehydes (A2) and
236 ketone (A3) are almost unchanged negative control.

237 **IgY effect on levels of IL-6 and TNF-α**

238 To evaluate the response of *F. nucleatum* stimulation on rats' TNF-α and IL-6, rat oral
239 cavity was incubated with *F. nucleatum*. After different concertation treatment of IgY, IL-6
240 and TNF- α were assessed by ELISA. The levels of the anti-inflammatory cytokine were
241 higher in the control group compared to IgY-treated subjects after 4 weeks treatment. As
242 shown in Figure 5, the concentration of TNF-α and IL-6 after the treatment of 10mg/ml (low-
243 dose) were significantly decreased (p<0.05) compared with control group. IL-6 reduced from
244 11.2ng/ml to 5.3ng/ml at high dose IgY and TNF-α reduced from 7.3ng/ml to 4.7ng/ml. The
245 concentration of IL-6 quite significantly (p<0.01) decreased in high-dose group, and the level
246 of TNF-α was significantly (p<0.01) lower than control group, and the level of TNF-α was
247 marker (p<0.01) lower than control group. Additionally, it was obvious that low-dose IgY

groups reduced the IL-6 and TNF-α level by about 7% and 9.6% compared to the level in the negative group.

Alveolar Bone Loss and Histopathological Analysis

The mouse model of periodontitis caused by *F. nucleatum* infection was used to investigate the effect of IgY. The results of X-Ray and Histological examination by H&E staining were showed in Fig.6. The results of X-ray showed that the gap between the teeth of the control group is large and the alveolar bone has a large gap between the teeth. After four weeks treatment of high-dose IgY, the gaps between teeth significantly reduce and alveolar bone is close to teeth. As shown in Fig. 6 B, there is a clear trend of decreasing alveolar bone resorption after different concentration of IgY. Moreover, disorganization of the connective tissue and remarkable inflammatory infiltration and were observed in Figure 6 C. A effective rehabilitation of periodontium, i including restoration of collagen fibers was detected in Figure 6 C. After 4-weeks' treatment, high-dose (40mg/ml) IgY significantly improved periodontal restoration.

Discussion

Many *in vitro* studies have investigated that special IgY has antimicrobial activity of series of oral bacteria, such as *Salmonella enteritidis* [22], *F. nucleatum* [23]. Most researches only investigated the inhibition of fungicide and metal ion in general medium. Saliva contains a complex mixing of protein, vitamin and ion, but the acquisition of saliva needs many volunteers and saliva has less reproducibility. In this *in vitro* study, the artificial saliva that has the similar components with natural saliva was used to simulate the human saliva that *F. nucleatum* grows in the oral cavity. With artificial saliva, it is easy to control macromolecular, ion and amino acids of the medium to manipulate their effect on *F. nucleatum* growth and

metabolism [18]. In this study, specific IgY significantly reduced amount of *F. nucleatum* (Fig.1). The IgY targeted 40- kDa outer membrane protein of *P. gingivalis* has been proved that IgY can significantly inhibit the coaggregation of *P. gingivalis* with other bacteria [15]. Thus, IgY may effectively bind cell surface components including outer membrane proteins or lipopolysaccharides to inhibit *F. nucleatum* growth [17]. The cell membrane fatty acid of *Fusobacteria* strains contains C14:0, C16:0, and cis-9 C16:1 which are the primary fatty acids [24]. In this study, IgY reduces the amount of fatty C16:0, C16:1 and C18:0, which may indicate that IgY acts on the metabolism of fatty acids to inhibit *F. nucleatum*. At the same time, the changes of short-chain fatty acids in saliva may also be related to the reduction of long-chain fatty acids.

VSCs compounds including H2S and CH3SH dominate halitosis [25]. After the treatment of IgY, odor index significantly decreased, which indicates IgY prevents halitosis in vivo (Fig.4). After the treatment of IgY, odor index significantly decreased, which indicates IgY prevents halitosis *in vivo* (Fig.4). Bacterial enzymes, L-cysteine desulfhydrase and METase, produce VSCs by digesting amino acids [26]. However, many amino acid catabolic enzymes situated in bacteria, which caused antibodies unable efficiently affect them. Inhibition of microbial growth by antibodies is another effective alternative method. In our study, IgY was able to significantly reduce VSCs by inhibiting the growth of bacteria. Zinc ions have two mechanisms of malodor inhibition by binding with H_2S and antimicrobial activity [27]. According to the reports, *F. nucleatum* produces VSC, which is related with Cdl (Fn1220) and Lcd (Fn0625) [28]. Cetylpyridinium chloride (CPC) has been founded that it can impress the cdl expression in *F. nucleatunm*. Meanwhile, CPC induces lcysteine desulfhydrase expression [29]. IgY may influence the expression of VSCs-related gene which was speculated.

295 This study used live *F. nucleatum* to imitate halitosis and periodontitis in mice oral
296 cavity. In oral cavity, VSCs are one of the source of bad breath; VOCs also are important
297 parts of halitosis. VOCs in breath dissolve in saliva influencing its composition. Human saliva
298 contains complex composition including peptides, proteins, and numerous volatile organic
299 compounds. Various oral bacteria use different systems to degrade nutrients that produces
300 various volatile organic compounds (VOCs). Several studies have investigated the volatile
301 organic compounds in saliva. They reported that the presence of six types of compounds
302 ketones, alcohols, aldehydes, aldehydes, esters and volatile acids [30]. The changes of
303 salivary VOCs composition may be an excellent indicator of halitosis through the treatment of
304 IgY. Short chains alcohols, ketones and esters are detected in our research. The methods of
305 saliva collection also may influence its composition. So, a non-stimulated saliva collection
306 approach was chosen in this study to avoid influence. Although rat oral cavity is different
307 from human, the changes of VOCs are similar to human. After the treatment of IgY, odor
308 index significantly decreased, which indicates IgY prevents halitosis. Volatile acids have a
309 stimulating odor. IgY effectively inhibit halitosis by reducing part of the ingredients of VOCs.
310 The model of rat may provide an effective method for development of anti-halitosis and anti-
311 periodontitis drugs or antibodies in the future.

312 In this model, mechanical trauma caused by ligature destroys the integrity of tissue to
313 induce an inflammatory response and periodontal ligament, which induces serious bone loss
314 [32]. *In vivo* study, periodontitis rat without IgY treatment have significant bone loss
315 compared with IgY treatment, accompanying collagen fiber disorder. Prior studies have noted
316 that the anti-*P. g* IgY in the periodontitis disease could decrease the probing depth [33]. There
317 are macrophages in dental pulp that are activated by TNF-α [34, 35]. Anaerobic bacteria
318 produce many metabolic end products including short-chain fatty Acids. Secondly, volatile

319 acids (eg, butyric, acetic, isovaleric and propionic) are important parts of halitosis. Butyric
320 and propionic acids have been proved that they have cytotoxicity to vero cells [36]. In this
321 study, butyric acid, propionic acid and caproic acid are detected using SPE-GC-MS. Butyric
322 acid is one of the major by-products of *F. nucleatum*. Butyric acid also can inhibit T-cell
323 proliferation and stimulate the monocytes and neutrophils to produce proinflammatory
324 cytokines [37] which increase the severity of periodontitis and halitosis. Our study is in
325 agreement with previous studies that increased cytokine IL-6 and TNF-α in response to *F.*
326 *nucleatum* [38]. Specific IgY targeting to *F. nucleatum* significantly inhibit cytokine, which is
327 may related to the reduction of butyric acid.

328 In conclusion, IgY induces systemic immune responses to *F. nucleatum* and VSCs in
329 dose-dependent manner *in vitro*. SPE-GC-MS revealed that specific IgY possibly acts on
330 mechanism *F. nucleatum*. The IgY has an effective impact on periodontal and halitosis
331 disease that caused by *F. nucleatum* in rat, which was further proved by its ability to decrease
332 alveolar bone loss, inflammation and VOCs. *F. nucleatum* has also been involved in the
333 pathogenesis of several diseases [39]. Most important, the study provides an effective strategy
334 for the treatment of disease caused by *F. nucleatum*.

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341 **Conflict of interest**

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Fig.1 The inhibitory effect of IgY on the growth of *F. nucleatum*

F. nucleatum were incubated in artificial saliva medium with high-dose (40 mg/ml) (■), mid-dose (20mg/ml) (●), low-dose(10mg/ml) (▲) and blank control (▼). Experience data are represented as mean ± S.D. of three individual experiments. * means $P < 0.05$, and ** means $P < 0.01$ as compared with the control.

Fig. 2 The fatty acids of different concentration IgY in artificial saliva

Experience data are represented as mean ± S.D. of three individual experiments. * means $P < 0.05$, and ** means $P < 0.01$ compared with the control.

Fig. 3 The effect of IgY on rat malodor induced by *F. nucleatum*

Mean ± S.D. of malodor levels as scored by two judges (with results presented on a semi-integer scale of (0-5) of three individual experiments. * means $P < 0.05$.

Fig. 4 The decrement of VOCs from oral saliva sample of rats by GC-MS analysis

Experience data are represented as mean ± S.D. of three individual experiments. * means $P < 0.05$, and ** means $P < 0.01$ compared with the control.

Fig.5 The changes of TNF-α and IL-6 in rat serum

Experience data are represented as mean ± S.D. of three individual experiments. * means P < 0.05, and ** means P < 0.01 compared with the control. The concentrations of TNF-α and IL-6 in 10mg/ml (low-dose) were significantly (P<0.05) decreased compared with control. The concentration of IL-6 in high-dose were lower than control (P<0.01).

Fig.6 The influence of IgY on alveolar bone loss and periodontium restoration in periodontitis rats.

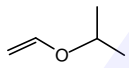
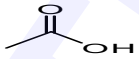
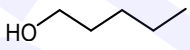
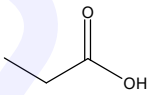
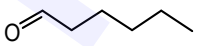
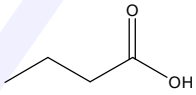
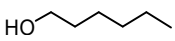
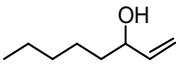
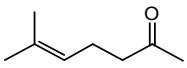
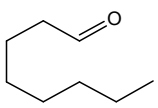
(A) Representative X-Ray images of alveolar bone in the rats. Black arrows indicate the gap between alveolar bone and teeth. (B) HE stains of the region that contains the first and the second upper molars. Black arrows indicate alveolar (C) HE stains of the periodontium. Black arrows indicate collagen fibers.

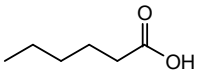
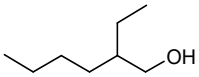
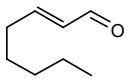
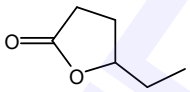
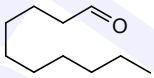
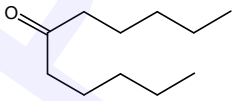
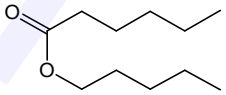
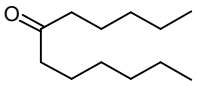
Table. 1 The VSCs production by *F. nucleatum* in different concentration IgY

Groups	Halimeter (mean mean ± SD, ppb)	*P*-value
Control	1921±99	
High-dose IgY	904±57	<0.01**
Mid-dose IgY	1233±52	<0.05 *
Low-dose IgY	1340±44	<0.05 *

Experience data are represented as mean ± S.D. of three individual experiments. * means P < 0.05, and ** means P < 0.01 compared with the control.

Table 2 Composition of VOCs of rat saliva

No.	Retention Time	CAS	Name	Structure	Classification
1	1.4	7664-41-7	Ammonia	NH_3	nitrogen compounds(C)
2	1.9	926-65-8	2-propane		others(E)
3	2.3	64-19-7	Acetic acid		carboxylic acid (A4-1)
4	3.8	71-41-0	pentanol		alcohol(A1-1)
5	4.9	79-09-4	Propionic acid		carboxylic acid (A4-2)
6	6.1	66-25-1	Hexanal		aldehyde(A2-1)
7	8.1	107-92-6	Butyric acid		carboxylic acid (A4-3)
8	9.1	111-27-3	n-Hexanol		alcohol(A1-2)
9	13.7	3391-86-4	1-Octen-3-ol		alcohol(A1-3)
10	14.6	110-93-0	Methyl isohexenyl ketone		ketone(A3-1)
11	15.1	124-13-0	Octyl aldehyde		aldehyde(A2-2)

12	15.5	142-62-1	Hexanoic acid		carboxylic acid (A4-4)
13	16.4	104-76-7	Isooctyl Alcohol		alcohol(A1-4)
14	17.5	2548-87-0	trans-2-Octen-1-al		aldehyde(A2-3)
15	18.3	695-06-7	gamma-hexalactone		ester(A5-1)
16	23.1	112-31-2	Decyl aldehyde		aldehyde(A2-4)
17	25.4	927-49-1	6-Undecanone		ketone(A3-2)
18	26	540-07-8	amyl caproate		ester(A5-2)
19	28	6064-27-3	dodecan-6-one		ketone(A3-3)

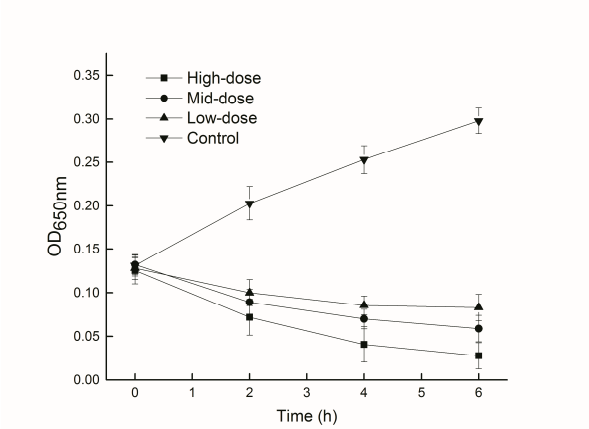


Figure 1

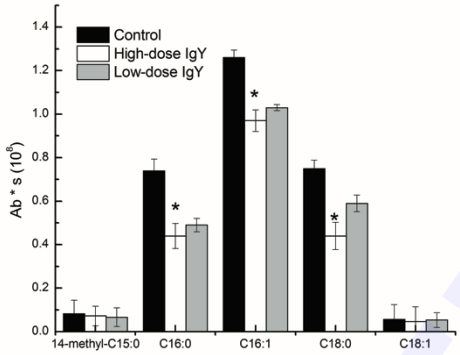


Figure 2

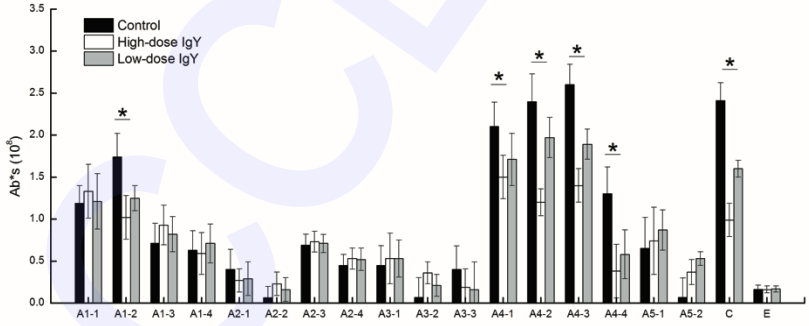


Figure 3

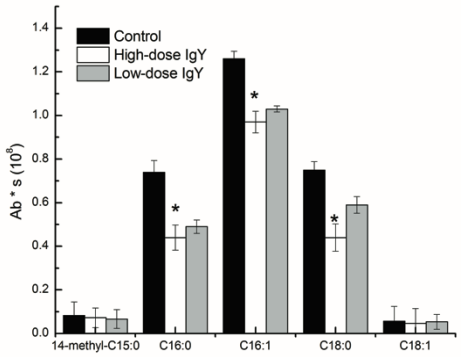


Figure 4

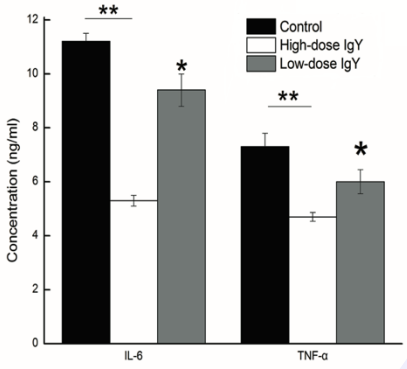


Figure 5

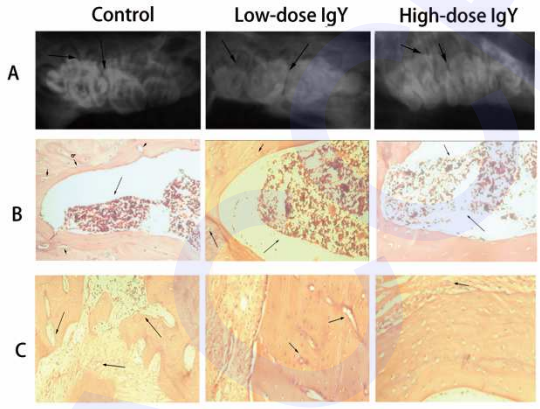


Figure 6