Sitagliptin Attenuates Porphyromonas Gingivalis Virulence and Inflammatory Response in Macrophage on Titanium

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1 **Sitagliptin Attenuates Porphyromonas Gingivalis Virulence**
2 **and Inflammatory Response in Macrophage on Titanium**

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Abstract

Background: In peri-implantitis, porphyromonas gingivalis and macrophage play central roles. The aim of this study was to detect the attenuating effect of an anti-diabetic drug sitagliptin on porphyromonas gingivalis virulence and inflammatory response in macrophage on titanium discs.

Materials and methods: Porphyromonas gingivalis and macrophage were cultured on titanium discs. Antibacterial and antibiofilm activities of sitagliptin were assessed and the morphology of porphyromonas gingivalis were observed by SEM. Bacterial early adhesion, aggregation, hemagglutination, hemolysis and porphyromonas gingivalis virulence factors mRNA expression were assessed to preliminarily investigate the mechanisms of action. Flow cytometry assay, qRT-PCR and Western Blot were used to assess the anti-inflammatory effect of sitagliptin on porphyromonas gingivalis lipopolysaccharide-stimulated macrophage.

Results: The present study demonstrated the inhibiting effect of sitagliptin on the growth, biofilm, phenotypic behavior and virulence factors of porphyromonas gingivalis and the protective effect on the porphyromonas gingivalis lipopolysaccharide-induced polarization in macrophage. And we also confirmed the anti-inflammatory effect of sitagliptin on the secretion of inflammation-related factors in macrophage by inhibiting the MAPK and AKT signaling pathways.

Conclusions: Sitagliptin possesses the attenuating effect on porphyromonas gingivalis virulence and inflammatory response in porphyromonas gingivalis lipopolysaccharide-stimulated macrophage on titanium.

Keywords: sitagliptin, porphyromonas gingivalis, macrophage, titanium, peri-implantitis.

Background

Based on the osseointegration theory put forward by Brånemark [1, 2], dental implantation has become a widely accepted and implemented treatment for tooth loss. Although reported high long-term survival rates were up to 96.1% after 10 years and 83.8% after 25 years [3, 4], implant failure may still occur. And in implant failure, peri-implantitis characterized by plaque-induced inflammation of the oral tissue surrounding the implants is a crucial element [5, 6]. Similar to the pathogenesis of periodontitis, peri-implantitis is the result of a continuum of microbial pressure, inflammation and tissue destruction [6].

In regard to microbial pressure, *Porphyromonas gingivalis* (*P. gingivalis*; Pg), the most representative strain involved in severe human periodontitis, appears in large numbers on titanium implants and produces several virulence factors in the process of peri-implantitis [7, 8]. Thus, attenuating virulence of *P. gingivalis* is a clinically valuable strategy to impede the progression of peri-implantitis.

As for plaque-induced inflammation, it was reported that a large number of inflammatory cells, particularly macrophages infiltrated peri-implantitis lesions [9]. Macrophage is regarded as a central player in immune-inflammatory processes due to its phagocytic capacity and high cellular plasticity [10] and its function and phenotype polarization is the focus of research on the inflammatory conditions contributing to diseases such as periodontitis, diabetes and peri-implantitis [11-13]. Stimulated by bacteria sub-products like lipopolysaccharides (LPS), macrophages present a M1 phenotype involving in pro-inflammatory reactions (e.g. secreting IL-1 β , IL-6, IL-8), while macrophages activated by alternative ways present a M2 phenotype and are associated with anti-inflammatory reactions [10]. Hence, a medication modulating M1/M2 status plays a significant role in attenuating inflammatory reaction of macrophage and counteracting the pathogenesis and destructive mechanisms of peri-implant diseases.

Sitagliptin, the first dipeptidyl-peptidase (DPP)-4 inhibitor, was approved and widely used as a glucose-lowering intervention for patients with type 2 diabetes [14]. Recently, more studies have reported the potential therapeutic effect of sitagliptin on other diseases including epilepsy, genital infections and COVID-19 [15-17]. Since studies have shown that DPP-4 is also expressed on the surface of macrophages [18, 19], sitagliptin may have an influence on the biological function of macrophages, thus regulating the inflammatory diseases in which macrophage involved.

P. gingivalis DPP-4 exhibits the similar substrate specificity to its human counterpart, hence sitagliptin could inhibit *P. gingivalis* DPP-4 as well [20-22]. And due to the suppressing effect of sitagliptin on quorum-sensing (QS) and X-prolyl dipeptidyl aminopeptidase which is similar to DPP-4, recent studies have determined that sitagliptin could attenuate the streptococcus mutans [23], *Serratia marcescens* [24] and *Pseudomonas aeruginosa* virulence [25]. However, to our knowledge, there is few study on the effects of sitagliptin on *P. gingivalis* in which QS and X-prolyl dipeptidyl aminopeptidase also play significant role.

Hence, it is reasonable to speculate that sitagliptin can be used as a potential agent to

control peri-implant inflammation by inhibiting *P. gingivalis* virulence and *P. gingivalis* LPS-induced inflammation of macrophage. To verify our hypothesis, the aim of this study was to detect the effect of sitagliptin on the virulence of *P. gingivalis*, the *P. gingivalis* LPS-induced polarization and inflammation related factors secretion of macrophage and the possible mechanism.

Materials and Methods

1 Titanium disc preparation

Commercial pure titanium discs (TA1 grade according to ISO 5832-2:1993) of 34mm and 15mm in diameter, and 1 mm in thickness were purchased from BaoJiXinNuo Ltd. (Shanxi, China). To simulate practical roughness, discs were sandblasted and acid-etched (SLA) as previously described [26]. Before cell or bacteria culturing, a SLA disc was sterilized and then placed into each well of a flat-bottomed 24-well or 6-well microplate.

2 Bacteria and culture conditions

Porphyromonas gingivalis ATCC 33277 was used in this present study and cultured in Tryptic Soy Broth (TSB; BD, NJ, USA) with 5 mg/ml of yeast extract (Oxoid, Hampshire, UK), 5 µg/ml of hemin (Solarbio, Beijing, China), 1µg/ml of Vitamin K (Alaadin, Shanghai, China) and 0.5 mg/ml of L-cysteine hydrochloride (Alaadin, Shanghai, China) at 37 °C under anaerobic conditions.

3 Antibacterial activities assay

P. gingivalis suspension was adjusted in TSB containing 1×10^7 colony-forming units (CFU)/mL with Sitagliptin (Topscience, Shanghai, China) prepared ranging from 2500 to 25 µg/ml or TSB as control. 1ml of the suspension was added in each well of a 24-well microplate to cover the SLA disc, and incubated for 48h. The MIC referred to the lowest concentration of sitagliptin inhibiting microorganism growth.

After incubation, 10µl suspension from each well was transferred to the Columbia blood plate medium (Luqiao Technology, Zhengzhou, China). The MBC was the lowest concentration of sitagliptin at which no colony occurs after incubation for 3–5 days under anaerobic conditions[27].

4 Antibiofilm activities assay

4.1 Biofilm formation assay

P. gingivalis biofilm formation was assayed by the crystal violet staining method [28]. *P. gingivalis* suspension (1×10^7 CFU/mL) with sitagliptin of different concentration was added in a 24-well microplate. After 48h of incubating, the cultures were gently discarded and the adherent biofilms on the SLA discs were rinsed for three times with phosphate buffered saline (PBS). Then, the adherent biofilms were incubated with anhydrous methanol for 15 min and then stained with 0.1% (w/v) crystal violet (CV)

for 15 min. After the excess CV was washed away with double distilled water, anhydrous methanol was added to dissolve the CV stained on biofilms. Finally, the anhydrous methanol was transferred to a 96-well microplate and the optical density (OD) values at the wavelength of 560 nm were recorded by using microplate reader. The minimum biofilm inhibition concentration (MBIC₅₀) was defined as the lowest concentration of sitagliptin that resulted in at least 50% inhibition of the biofilm formation compared with the control.

4.2 Biofilm reduction assay

The effect of sitagliptin on *P. gingivalis* biofilm reduction was also assayed by CV staining [29]. To form mature biofilms, *P. gingivalis* suspension (1×10^7 CFU/mL) was grown in a 24-well microplate for 48h.

After removing the supernatant, the non-adherent bacteria were gently rinsed off with PBS. Then, TSB medium with sitagliptin of different concentration was added into the microplate for 24h anaerobically. The biomass and metabolic activity were determined with the CV assay as described above. Minimum biofilm reduction concentration (MBRC₅₀) was defined as the lowest concentration of sitagliptin required to eradicate the preformed biofilm by at least 50%.

4.3 Biofilm viability assay

The effects of sitagliptin on *P. gingivalis* biofilm viability were tested with a CCK8 kit (Dojindo, Shanghai, China). First, the biofilm was treated in the same way as in the biofilm reduction assay. Subsequently, 1mL TSB medium mixed with 10% CCK-8 reagent was added into each well of 24-well microplate. After incubation of 2h, supernatant was transferred to a 96-well microplate for analysis at 450 nm (630 nm as reference). The sessile MIC (SMIC₅₀) referred to the lowest drug concentration resulting in at least 50% reduction compared with that of control.

5 Morphological observation by scanning electron microscopy (SEM)

P. gingivalis suspension (1×10^7 CFU/mL) with TSB and sitagliptin dilution of MIC and MBC was added in 24-well microplate. After incubation of 48h, the SLA discs were washed with PBS three times and then fixed with 2.5% (v/v) glutaraldehyde for 5h. The discs were washed with PBS, followed by a dehydration with a serial concentration (75, 80, 90, 95 and 100%) of ethanol solutions. Samples were then critical-point-dried and coated with gold. After that, *P. gingivalis* was photographed by SEM.

6 Effect of sitagliptin at sub-MIC on *P. gingivalis* virulence

6.1 bacterial growth assay

According to Abbas and Hegazy [24], the effect of sitagliptin at sub-MIC on the growth of *P. gingivalis* was detected. After incubation with or without sitagliptin at sub-MIC as described above, 100 μ l suspension from each well was transferred to a 96-well microplate and then measured at the wavelength of 600nm.

6.2 Early adhesion to titanium

The SLA discs were immersed in *P. gingivalis* suspension of 1×10^8 CFU/mL with sitagliptin of sub-MIC and incubated for 4h anaerobically. To visually observe and quantificationally measure the *P. gingivalis* early adhered to the SLA discs, SEM and CCK8 method were used as described above.

6.3 Bacterial aggregation

As described previously [30], *P. gingivalis* aggregation was measured with minor modifications. *P. gingivalis* were incubated with sitagliptin of sub-MIC overnight anaerobically. Harvested by centrifugation, the bacteria were rinsed and re-suspended with PBS to OD₆₀₀=1.0. The suspension was monitored for autoaggregation (decrease in absorbance as the cells clumped) after 3h.

6.4 Bacterial hemagglutination

Hemagglutination assay was performed as described previously [30, 31], with modifications. 24h cultures of *P. gingivalis* with sitagliptin of sub-MIC were harvested, centrifuged, and re-suspended. The bacteria suspension was serially diluted (1:2 to 1:128) in PBS and mixed with sitagliptin of sub-MIC. Then 100 μ L of the mixture was added into each well of a 96-well plate. After the sheep defibrinated blood (Solarbio, Beijing, China) was centrifugated and rinsed with PBS three times, the erythrocytes were harvested and diluted to 5% (v/v). Subsequently, 100 μ L of sheep erythrocytes suspension were added to the bacteria suspension and incubated at room temperature for 3h. Finally, hemagglutination was detected visibly.

6.5 Bacterial hemolysis

The quantification of bacterial hemolysis was performed as described [32] with modification. Overnight cultures of *P. gingivalis* were adjusted to OD₆₀₀=1.5. Sheep erythrocytes were washed until no hemoglobin can be detected visibly in the supernatant. Then the sheep erythrocytes were diluted to 2% (v/v) and mixed with an equal volume of bacteria suspension with or without sitagliptin. Control samples were incubated with PBS (negative control) or 1% triton X-100 (positive control). The mixture was added to cover the SLA disc in a 24-well plate and incubated at 37 °C for 18 h. The hemolysis was quantified at wavelength of 540 nm.

6.6 Effect on the expressions of virulence factors

To determine the effect of sitagliptin of sub-MIC on the expression of *P. gingivalis* virulence factors, reverse transcription-quantitative polymerase chain reaction (RT-PCR) was performed as follows. The sitagliptin of sub-MIC was added to bacterial cultures (OD₆₀₀=0.8) prior to anaerobic culturing at 37 °C for 24 h. Then the total RNA of bacteria was extracted by Trizol reagent (Takara, Dalian, China), followed by measurement of RNA concentration by Nanodrop and reverse

transcription by PrimeScript RT reagent Kit (Takara, Dalian, China). Finally, PCR was performed with 10 μ L of a mixture containing 2 μ L of the cDNA template, 2 μ L of double distilled water, 5 μ L of SYBR Green Realtime PCR Master Mix (Toyobo, Shanghai, China) and 0.5 μ L of each primer, under the following conditions: 2 min at 95 °C, 5s at 95 °C and 30 s at 60 °C for 40 cycles, 5 s at 95 °C, 5 s at 65 °C, 5 s at 95 °C. The forward and reverse primer sequences are shown in Table1.

7 Cell culture

Obtained from the China Center for Type Culture Collection, Wuhan, China, RAW 264.7 was used in this present study and cultured in high glucose DMEM (Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) at 37°C in a 5% CO₂ incubator (Thermo, USA). For different experiments, RAW 264.7 was cultured in a 24-well or 6-well plate covering the SLA discs.

8 Cytotoxicity Assay

RAW 264.7 was seeded in a 24-well plate at a density of 1×10^5 cells/well and then cultured for 24h, with sitagliptin or sitagliptin + 5 μ g/ml Pg-LPS (Invivogen, Shanghai, China). Then the cell viability was determined by using CCK8 kit as described above.

9 Flow cytometry assay

Differently treated RAW264.7 cells (10⁶/sample) were washed twice in PBS containing 2% FBS and then incubated with fluorescently labeled antibodies CD86-APC (biolegend, USA) for 30 min at 4°C. Subsequently, samples were washed twice and fixed and permeated with Fix/Perm Buffer (BD Pharmingen, USA). After that, cells were incubated with CD206-PerCP/Cy5.5 (biolegend, USA) as described above. Cells were washed twice prior to flow cytometry.

10 RT-PCR assay

RT-PCR assay was used to analyse gene expression at mRNA level. Differently treated cells were harvested and rinsed twice with PBS. Then the RNA isolation and qRT-PCR assay was performed as described above. The forward and reverse primer sequences are shown in Table2.

11 ELISA assays

For cytokine gene-expression analysis at protein level, ELISA kit (Neobioscience, Shenzhen, China) were used to measure IL-1 and IL-6 in the supernatant following the manufacturer's protocol.

12 Western Blot Analysis

Differently treated cells were harvested, washed twice with cold PBS and then lysed

with RIPA buffer (Servicebio, Wuhan, China) on ice. After sonication and centrifugation, the protein concentrations of samples were determined with BCA protein quantitative detection kit (Beyotime, Shanghai, China) and normalized. Subsequently, denatured lysate proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were then blocked and incubated with primary antibodies (Servicebio) overnight at 4°C, prior to incubating with secondary antibody (Servicebio) and visualization reagent. The chemiluminescence was detected with Image Studio System (LICOR, Lincoln, NE, USA). Immunoreactive bands were quantified with Image J analysis.

13 Statistical Analysis

GraphPad Prism 8.0.1 software was used for statistical analyses. All results are presented as means± standard deviation (SD). Ordinary one-way analysis of variance (ANOVA) was used to compare the differences between parameters in each group, and $p < 0.05$ were considered significant.

Results

1 The antimicrobial assay of sitagliptin against *P. gingivalis*

The activity of sitagliptin against planktonic *P. gingivalis* and *P. gingivalis* biofilm was determined by broth microdilution method. Results of the susceptibility assay of planktonic *P. gingivalis* to sitagliptin are shown in Table 3. The MIC and the MBC values of sitagliptin were 250 and 2500 µg/ml, respectively.

As shown in Figure 1 (A-C) and Table 3, sitagliptin showed its effect on the formation, reduction and viability of *P. gingivalis* biofilm. The MBIC₅₀, MBRC₅₀ and SMIC₅₀ of sitagliptin was 500, 2000 and 1000 µg/ml, respectively.

To explore the antibacterial mechanism of sitagliptin against *P. gingivalis*, the morphological changes of *P. gingivalis* treated with sitagliptin on SLA discs was observed by SEM. In the control group, the cells observed were plump and cell membranes remained clearly intact (Figure 1D). Treated with sitagliptin of MIC, *P. gingivalis* cell membrane was shrunken (Figure 1E). And treated with sitagliptin of MBC, perforation of cell membrane was observed and the cell structures were totally ruptured (Figure 1F). Thus, cell membrane may be a sensitive target for sitagliptin against *P. gingivalis*.

2 Effect of sitagliptin at sub-MIC on *P. gingivalis*

2.1 *P. gingivalis* growth assay

Following 48-hour incubation, the OD₆₀₀ value of *P. gingivalis* suspension without or with sitagliptin of sub-MIC was measured. No statistically significant difference was found between the turbidity of the *P. gingivalis* suspension without or with sitagliptin (up to 50 µg/ml, 1/5MIC), demonstrating the lack of effect of sitagliptin (1/5 MIC) on bacterial growth (Figure 2).

2.2 Early adhesion to titanium and bacterial aggregation

The early adhesion of *P. gingivalis* to SLA discs was visually observed by SEM. In the control group, more *P. gingivalis* cells observed adhered to the SLA discs and clumped together (Figure 3A), while fewer cells adhered to the discs and distributed separately after incubating with sitagliptin (Figure 3B).

To quantificationally measure the *P. gingivalis* early adhered to the SLA discs, CCK8 method were used. As shown in Figure 3C, sitagliptin of 50 µg/ml significantly reduced *P. gingivalis* early adhesion to the SLA discs. And to determine the *P. gingivalis* bacterial aggregation, the OD_{600nm} value of the bacterial suspension was recorded. As shown in Figure 3D, the aggregation rate was 57.33% ± 4.04% after incubation while the rate decrease to 47.67% ± 4.73% after incubating with sitagliptin.

2.3 Bacterial hemagglutination and hemolysis

As shown in Figure 4A, bacterial hemagglutination was detected visibly up to the bacteria dilution of 1:8 after incubating without sitagliptin or with sitagliptin of 25 µg/ml, whereas hemagglutination was observed up to the bacteria dilution of 1:4 with 50 µg/ml of sitagliptin indicating the effect of sitagliptin at 50 µg/ml on hemagglutination activity.

And bacterial hemolysis, as shown in Figure 4B, decreased significantly from 46.23% ± 2.05% without sitagliptin to 31.07% ± 3.02% with 50 µg/ml of sitagliptin which demonstrated that effect of sitagliptin at sub-MIC on bacterial hemolytic activities.

2.4 Expressions of virulence factors

As shown in Figure 5, compared with *P. gingivalis* incubated without sitagliptin, sitagliptin at 50 µg/ml significantly decreased the gene expressions of all tested virulence factors including hemolysin (hem), fimbriae (fimA), arginine-specific gingipains (rgpA), lysine-specific gingipain (kgp) and ferritin (ftn).

3 Effect of sitagliptin on inflammatory response in RAW 264.7

3.1 Cytotoxicity assay

To determine the cytotoxicity of sitagliptin at different doses, the CCK-8 assay was performed after RAW264.7 cells incubated without or with sitagliptin (10, 25, 50, 75 and 100 µg/ml) for 24 h. As shown in Figure 6A, sitagliptin had no effect on cells viability at concentrations of up to 50 µg/ml.

And to the cytotoxicity of sitagliptin with Pg-LPS stimulation, viability of cells treated with sitagliptin in the absence or presence of 5 µg/ml of Pg-LPS for 24h was detected. The CCK-8 assay demonstrated that no significant difference of cell viability between the control group (without sitagliptin and LPS) and the treated

group was found (Figure 6B).

3.2 Protective effect of sitagliptin against the polarization of M1 cells

RAW264.7 cells were treated with or without 50 µg/ml of sitagliptin in the absence or presence of 5 µg/ml of Pg-LPS. The ratio of CD86/CD206 of the control group was 0.75 ± 0.03 , while that of the treatment with 5 µg/ml of Pg-LPS was increased to 2.05 ± 0.05 showing that Pg-LPS promoted the polarization of M1 cells (Figure 7 A,B,D). Compared to the treatment with Pg-LPS, treatment with Pg-LPS and 50 µg/ml of sitagliptin decreased the ratio to 1.59 ± 0.12 indicating the protective effect of sitagliptin against the polarization of M1 cells (Figure 7 C,D).

3.3 Attenuating effect of sitagliptin on the expression of inflammation-related cytokine in response to *P. gingivalis* LPS

To determine the effect of sitagliptin on proinflammatory cytokine expression, ELISA and RT-PCR assay were performed. At transcriptional level, the expression of proinflammatory cytokine including IL-1 β , IL-6, TNF- α , iNOS, IL12-P35 and IL12-P40 was significantly increased by 5 µg/ml of Pg-LPS, while it decreased significantly after treatment with 50 µg/ml of sitagliptin (Figure 8A). At translational level, treatment with sitagliptin and LPS also significantly downregulated the enhancement of IL-1 β and IL-6 stimulated by LPS (Figure 8 B,C).

3.4 Inhibiting effect of sitagliptin on activation of MAPK and AKT signaling pathways

To investigate the underlying mechanism of anti-inflammatory effect of sitagliptin, the effect of sitagliptin on the MAPK and AKT signaling pathways was examined. As shown in figure 9, 5 µg/ml of Pg-LPS stimulation significantly increased the phosphorylation of p38 MAPK, AKT and ERK. However, treatment with 50 µg/ml of sitagliptin significantly inhibited phosphorylation of p38 , AKT and ERK indicating that sitagliptin suppressed the MAPK and AKT signaling pathways.

Discussion

Peri-implantitis is a plaque-induced inflammatory disease that may lead to dental failure [6]. For the treatment of peri-implantitis, drug therapy, mostly with antibiotics, is an essential adjunctive method [33]. However, considering the possible drug resistance and side effects of antibiotics, more and more studies have focused on the potential alternatives. Current evidence pointed out that diabetes was an important risk factor of peri-implantitis [34-36], thus in this present study, we carried out the *in vitro* experiment to explore the possible application of sitagliptin, a traditional glucose-lowering agent for type 2 diabetes patients, in attenuating the *P.gingivalis* virulence and subsequent inflammatory response in peri-implantitis.

P.gingivalis is a key periodontal pathogen and strongly associated with peri-implantitis [37]. In the present study, we determined that sitagliptin not only inhibited the growth of planktonic *P. gingivalis* but also impaired *P. gingivalis* biofilm

by inhibiting biofilm formation, eliminating established biofilm, and reducing biofilm viability, which may be on the ground of the inhibiting effect of sitagliptin on X-prolyl dipeptidyl aminopeptidase that is similar to DPP-4 and plays a role in virulence of *P.gingivalis* [23]. And by SEM, we observed the perforation of *P. gingivalis* cell membrane treated with sitagliptin of MBC, hence we speculated that the cell membrane may be a sensitive target for sitagliptin against *P. gingivalis*.

Besides, we studied the effect of sitagliptin at sub-MIC on the phenotypic behavior of *P. gingivalis*. For non-motile bacteria like *P. gingivalis*, adhesion is the initial step for the biofilm formation [38]. The 50µg/ml (1/5MIC) of sitagliptin significantly reduced early adhesion of *P. gingivalis* compared to the control. This may be due to the anti-QS activity of triazole derivatives including sitagliptin, which controls diverse physiological functions of bacteria such as adhesion, hemolysis, production of biofilm and swimming. Aggregation is a process through which a strain within the biofilm produces polymers to integrate genetically identical strains and is also a significant prerequisite of biofilm formation [40, 41]. Treated with sitagliptin, the *P. gingivalis* observed by SEM distributed more separately and the aggregation rate detected by microplate assay decreased significantly determining the effect of sitagliptin on *P. gingivalis* aggregation.

In the present study, we also investigated the effect of sitagliptin on hemagglutination and hemolysis of *P. gingivalis*. Different to other bacteria, *P. gingivalis* cannot take in iron and biosynthesize heme for the lack of a siderophore scavenging system and the required enzymes, while it obtains heme directly from erythrocytes adopting alternative mechanisms [42]. The mechanisms involve the hemagglutinating and hemolytic activities of *P.gingivalis*, which is a feature distinguishing this organism from other black-pigmented anaerobes [43]. Our results determined the inhibiting effect of sitagliptin at 50 µg/ml on the hemagglutination and hemolysis of *P.gingivalis*, indicating that sitagliptin may play a significant role in attenuating virulence of *P. gingivalis* by controlling the heme acquisition of *P. gingivalis*.

To detect the effect of sitagliptin on the gene expressions related to *P. gingivalis* virulence, our study performed real-time PCR analysis. Our results demonstrated that sitagliptin at 50 µg/ml significantly decreased the gene expressions of all tested virulence factors including arginine-specific gingipains (*rgpA*), lysine-specific gingipain (*kgp*), fimbriae (*fimA*), hemolysin (*hem*) and ferritin (*ftn*). Among them, *rgpA* and *kgp* genes are involved with gingipain, which has been extensively studied and are considered to be responsible for bacterial adhesion, hemagglutination and hemolytic activities, host tissue destruction by inducing matrix metalloproteinase production and stimulation inflammatory response [44-46]. *FimA* gene is responsible for adhesion, co-aggression and interaction with TLR-2 receptor mediating pro-inflammatory cytokines production [38, 47]. *Hem* is related to hemolysis activity facilitating the acquisition of heme from erythrocytes, which is necessary for bacterial growth [30]. And *ftn* gene involves with intracellular Fe²⁺ storage and particularly significant for *P. gingivalis* to survive under iron deprived conditions [48, 49]. Therefore, the PCR results further determined that sitagliptin inhibited the above *P. gingivalis* virulence at the transcriptional level.

In the inflammatory progress of peri-implantitis, the polarization of macrophage plays a considerable role [10, 12]. It is reported that macrophages reveal a higher expression for M1 phenotype and accordingly a higher M1/M2 ratio at peri-implantitis sites [12]. Thus in the present study, we detected the effect of sitagliptin on polarization of RAW264.7 via flow cytometry assay. On account of that CD86 is the characteristic marker of M1 macrophages while CD206 is primarily expressed by M2 macrophages [50], CD86 and CD206 were selected as M1 and M2 markers for flow analysis. It is generally accepted that macrophages activated by bacteria sub-products LPS present a M1 phenotype [10], which is consistent with our result that RAW264.7 cells presented a higher expression of M1 treated with Pg-LPS. However, RAW264.7 treated with Pg-LPS and sitagliptin presented a lower expression of M1 and a lower M1/M2 ratio showing the protective effect of sitagliptin on the macrophage polarization.

Macrophages presenting a M1 phenotype are associated with pro-inflammatory responses, tissue destruction, and pro-inflammatory cytokine production [10]. Thus we further detected the effect of sitagliptin on the inflammation-related cytokine expression of RAW264.7 stimulated by Pg-LPS and found that sitagliptin significantly downregulated the enhancement of IL-1 β , TNF- α , IL-6 stimulated by LPS at transcriptional level and IL-1 β and IL-6 at translational level. It is demonstrated that peri-implantitis sites were associated with a significant increase in levels of IL-1 β and TNF- α compared to healthy implants [51]. IL-1 β is mainly produced in macrophages and regulates the degradation of collagenase activity and extracellular matrix components of the plasminogen system in inflammation [52]. And TNF- α induces fibroblast apoptosis and reduction of the repair capacity of the peri-implant tissue [53]. Besides, IL-6, another one of the most investigated pro-inflammatory cytokines between healthy and diseased peri-implant tissues, is conspicuous in chronic peri-implant inflammation, leading to osteoclastic activation and peri-implant bone loss [54]. We also found that sitagliptin decreased the mRNA expression of iNOS, IL-12 p35 and IL-12 p40. iNOS is the M1 macrophage marker gene and its activation results in further propagation of inflammatory responses [55]. And IL-12, composed of p35 and p40 subunits, is processed by macrophages correlating with bacterial clearance [56]. IL-12 is not toxic itself but prompts a neutrophil response that determined the severity of tissue damage [57, 58], therefore our results may suggest that sitagliptin can control excessive levels of IL-12.

Studies determined the important roles of MAPK and AKT signaling pathways in peri-implantitis [59-61]. Our study showed that *P. gingivalis* LPS significantly enhanced the phosphorylation of p38 MAPK, AKT and ERK while the treatment of sitagliptin decreased the phosphorylation, suggesting that sitagliptin may exert anti-inflammatory effects by inhibiting the MAPK and AKT signaling pathways.

Taken together, our study demonstrated the attenuating effect of sitagliptin on the growth, phenotypic behavior and virulence factors of *P. gingivalis* and the protective effect on the *P. gingivalis* LPS-induced polarization in macrophage. And we also confirmed the anti-inflammatory effect of sitagliptin on the secretion of inflammation-related factors in macrophage stimulated by *P. gingivalis* LPS by inhibiting the MAPK and AKT signaling pathways. And it is the first *in vitro* study on

the inhibiting effect of sitagliptin on *P.gingivalis* virulence and inflammatory response in *P.gingivalis* LPS-stimulated macrophage on titanium suggesting the potential therapeutic role of the anti-diabetic drug sitagliptin in peri-implantitis, particularly in patients with type 2 diabetes. However, the above effects of sitagliptin surrounding implant *in vivo* or in a high-glucose environment, need to be further studied.

Competing interests

The authors declare that they have no competing interests.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

W.T., M.D., S.Z., H.J.: conception and design; W.T., S.Z.: experiments and/or data analysis; M.D.: clinical consultancy; H.J.: intellectual input and supervision; W.T.: writing—original draft; S.Z.: writing—review and editing.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. Additional data will be available upon request from corresponding author.

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Not applicable.

Author information

Shuang Zhang and Han Jiang share the correspondence authors and both contributed equally to this study.

Table

Table1. Primer sequences used for transcription analysis of *P. gingivalis* virulence-associated genes.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
16S rRNA	CGGGAATAACGGGCGATACG	TACCGAACAACCTACGCACC
Kgp	ATGTTGCCTTCCGCCACTTCG	CTCCGTTACGCCTGATGCTATCTG
FimA	TTGTTGGGACTTGCTGCTCTTG	TTCGGCTGATTTGATGGCTTCC
Hem	ACGAAGCCTTGTTCTCCTCA	CAATGAATATGCCGGTTTCC
RgpA	CCGAGCACGAAAACCAA	GGGGCATCGCTGACTG
Ftn	GCGTGGCGGCGAGGTGAAG	CGGAAGCAGCCCTTACGACAGC

Table2. Primer sequences used for transcription analysis of RAW264.7 inflammation-related cytokine genes.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
GAPDH	TGGAAAGCTGTGGCGTGAT	GTCATCATACTTGGCAGGTTTCT
IL-1 β	AGGAGAACCAAGCAACGACA	CTTGGGATCCCACTCTCCAG
IL-6	ACAAGTCCGGAGAGGAGACT	AATTGCCATTGCACAACCTCTT
iNOS	GGAGCATCCCAAGTACGAGT	CCAATCTCGGTGCCCATGTA
IL-10	CAGTACAGCCGGAAGACAA	TGGCAACCCAAGTAACCCTTA
IL-12 p40	ACAGCACCAGCTTCTTCATCAG	TCTTCAAAGGCTTCATCTGCAA
IL-12 p35	CCAAGGTCAGCGTTCCAACA	AGAGGAGGTAGCGTGATTGACA

Table3. The antimicrobial assay of sitagliptin against *P. gingivalis*

Drug (μ g/ml)	Planktonic <i>P. gingivalis</i>		Biofilm of <i>P. gingivalis</i>		
	MIC	MBC	MBIC ₅₀	MBRC ₅₀	SMIC ₅₀
Sitagliptin	250	2500	500	2000	1000

Figure Legend

Figure1. Antibiofilm activity of sitagliptin and morphological changes of *P. gingivalis*. (A) After 48h of incubation with different concentrations of sitagliptin, a crystal violet staining assay was conducted and the OD₅₆₀ value were recorded. (B) Mature biofilm was treated with sitagliptin for 24h followed by a crystal violet staining assay and recordation of OD₅₆₀ value. (C) Mature biofilm was incubated with sitagliptin for 24 h followed by a CCK8 assay and recordation of OD₄₅₀ value. Data from three independent experiments were shown as mean $\pm$ standard deviation (SD). (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with *P. gingivalis* incubated without sitagliptin). (D-F) Morphological changes of *P. gingivalis* were observed by SEM. (D) Cells observed in the control group were plump and cell membranes remained clearly intact. (E) Treated with sitagliptin of MIC, cell membrane was shrunken (red arrows). (F) Treated with sitagliptin of MBC, perforation of cell membrane (red arrows) was observed and the cell structures were totally ruptured.

Figure2. Effect of sitagliptin at sub-MIC on *P. gingivalis* growth. Following 48-hour incubation, the OD₆₀₀ value of *P. gingivalis* suspension without or with sitagliptin of sub-MIC was measured. Data were expressed as mean $\pm$ SD. **P < 0.01, ***P < 0.001 compared with *P. gingivalis* incubated without sitagliptin.

Figure3. Effect of sitagliptin at sub-MIC on *P. gingivalis* early adhesion and aggregation. (A,B) The SLA discs were immersed in *P. gingivalis* suspension with 0, 25 and 50 μ g/ml of sitagliptin and then incubated for 4h anaerobically. The early adhered *P. gingivalis* (red arrows) to SLA discs was visually observed by SEM. (A) In the untreated group, more *P. gingivalis* cells observed adhered to the SLA discs and clumped together, (B) while fewer cells adhered to the discs and distributed separately in the treatment with sitagliptin of 50 μ g/ml. (C) A CCK8 assay was performed and data were shown as mean $\pm$ SD. (D) *P. gingivalis* were incubated with sitagliptin of sub-MIC overnight anaerobically. Harvested by centrifugation, the bacteria were rinsed and re-suspended. The OD_{600nm} value of the suspension was recorded after 3h. The percentage of aggregation was calculated by the following equation: $\text{Aggregation rate} = (\text{OD}_{\text{Initial}} - \text{OD}_{3\text{h}}) / \text{OD}_{\text{Initial}} \times 100\%$. *P < 0.05, **P < 0.01 compared with *P. gingivalis* incubated without sitagliptin.

Figure 4. Effect of sitagliptin at sub-MIC on bacterial hemagglutination and hemolysis. (A) Overnight cultures of *P. gingivalis* with sitagliptin were harvested, centrifuged, and re-suspended. The bacteria suspension were serially diluted and mixed with sitagliptin of sub-MIC and erythrocytes suspension. After incubation for 3h, hemagglutination was detected visibly. (B) After washing and diluting, sheep erythrocytes were added in bacteria suspension with or without sitagliptin. The OD₅₄₀ value was recorded. ***P < 0.001 compared with *P. gingivalis* incubated without sitagliptin.

Figure 5. Effect of sitagliptin at sub-MIC on the expressions of virulence factors. The sitagliptin of sub-MIC was added to bacterial cultures (OD₆₀₀ = 0.8) prior to anaerobic culturing at 37 °C for 24 h. Transcriptional levels of the virulence genes of *P. gingivalis* were detected by real time-PCR. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with *P. gingivalis* incubated

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Figure 6. Cytotoxicity assay.(A) Viability of cells treated with different concentrations of sitagliptin and (B) with sitagliptin in the absence or presence of Pg-LPS of 5 µg/ml for 24h was detected by CCK8 .**P < 0.01, ****P < 0.0001 compared with untreated group.

Figure 7. Protective effect of sitagliptin on macrophages polarization. (A) Flow cytometry assay of macrophages polarization of the untreated group; (B) flow cytometry assay of macrophages polarization of the treatment with 5 µg/ml of Pg-LPS; (C) flow cytometry assay of macrophages polarization of the treatment with 5 µg/ml of Pg-LPS and 50 µg/ml of sitagliptin; (D) analysis of the ratio of M1/M2. Data were expressed as mean $\pm$ SD. *P < 0.05 and ***P < 0.001 compared with cells treated with Pg-LPS.

Figure 8. Sitagliptin attenuates the expression of inflammation-related cytokine in response to P. gingivalis LPS. (A) Relative levels of IL-1 β , IL-6, TNF- α , iNOS, IL12-P35 and IL12-P40 mRNA determined by qPCR. (B) Proteins levels of IL-1 β and (C) IL-6 in supernatants measured by ELISA. (B,C) Data were expressed as mean $\pm$ SD. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with cells treated with Pg-LPS.

Figure 9. Sitagliptin Inhibits Activation of MAPK and AKT Signaling Pathways. RAW264.7 cells were treated with or without 50 µg/ml of sitagliptin in the absence or presence of 5 µg/ml of Pg-LPS. (A) Cell lysates were subjected to Western blot analysis. (B) The band intensities were quantified by Image J analysis. Data were expressed as mean $\pm$ SD. * p < 0.05 compared with treatment with Pg-LPS. ** p < 0.05 compared with Group1.

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Figures

Figure 1

Antibiofilm activity of sitagliptin and morphological changes of *P. gingivalis*. (A) After 48h of incubation with different concentrations of sitagliptin, a crystal violet staining assay was conducted and the OD560 value were recorded. (B) Mature biofilm was treated with sitagliptin for 24h followed by a crystal violet staining assay and recordation of OD560 value. (C) Mature biofilm was incubated with sitagliptin for 24 h followed by a CCK8 assay and recordation of OD450 value. Data from three independent experiments were shown as mean $\pm$ standard deviation (SD). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with *P. gingivalis* incubated without sitagliptin). (D-F) Morphological changes of *P. gingivalis* were observed by SEM. (D) Cells observed in the control group were plump and cell membranes remained clearly intact. (E) Treated with sitagliptin of MIC, cell membrane was shrunken (red arrows). (F) Treated with sitagliptin of MBC, perforation of cell membrane (red arrows) was observed and the cell structures were totally ruptured.

Figure 2

Effect of sitagliptin at sub-MIC on *P. gingivalis* growth. Following 48-hour incubation, the OD600 value of *P. gingivalis* suspension without or with sitagliptin of sub-MIC was measured. Data were expressed as mean $\pm$ SD. **P < 0.01, ***P < 0.001 compared with *P. gingivalis* incubated without sitagliptin.

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Effect of sitagliptin at sub-MIC on *P. gingivalis* early adhesion and aggregation. (A,B) The SLA discs were immersed in *P. gingivalis* suspension with 0, 25 and 50 µg/ml of sitagliptin and then incubated for 4h anaerobically. The early adhered *P. gingivalis* (red arrows) to SLA discs was visually observed by SEM. (A) In the untreated group, more *P. gingivalis* cells observed adhered to the SLA discs and clumped together, (B) while fewer cells adhered to the discs and distributed separately in the treatment with sitagliptin of 50 µg/ml. (C) A CCK8 assay was performed and data were shown as mean ± SD. (D) *P. gingivalis* were incubated with sitagliptin of sub-MIC overnight anaerobically. Harvested by centrifugation, the bacteria were rinsed and re-suspended. The OD_{600nm} value of the suspension was recorded after 3h. The percentage of aggregation was calculated by the following equation: Aggregation rate = (OD_{Initial} - OD_{3h}) / OD_{Initial} × 100%. *P < 0.05, **P < 0.01 compared with *P. gingivalis* incubated without sitagliptin.

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Effect of sitagliptin at sub-MIC on bacterial hemagglutination and hemolysis. (A) Overnight cultures of *P. gingivalis* with sitagliptin were harvested, centrifuged, and re-suspended. The bacteria suspension were serially diluted and mixed with sitagliptin of sub-MIC and erythrocytes suspension. After incubation for 3h, hemagglutination was detected visibly. (B) After washing and diluting, sheep erythrocytes were added in bacteria suspension with or without sitagliptin. The OD540 value was recorded. *** $P < 0.001$ compared with *P. gingivalis* incubated without sitagliptin.

Figure 5

Effect of sitagliptin at sub-MIC on the expressions of virulence factors. The sitagliptin of sub-MIC was added to bacterial cultures (OD600 = 0.8) prior to anaerobic culturing at 37 °C for 24 h. Transcriptional levels of the virulence genes of *P. gingivalis* were detected by real time-PCR. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with *P. gingivalis* incubated without sitagliptin.

Figure 6

Cytotoxicity assay.(A) Viability of cells treated with different concentrations of sitagliptin and (B) with sitagliptin in the absence or presence of Pg-LPS of 5 µg/ml for 24h was detected by CCK8 .**P < 0.01, ****P < 0.0001 compared with untreated group.

Figure 7

Protective effect of sitagliptin on macrophages polarization. (A) Flow cytometry assay of macrophages polarization of the untreated group; (B) flow cytometry assay of macrophages polarization of the treatment with 5 µg/ml of Pg-LPS (C) flow cytometry assay of macrophages polarization of the treatment with 5 µg/ml of Pg-LPS and 50 µg/ml of sitagliptin; (D) analysis of the ratio of M1/M2. Data were expressed as mean ± SD. *P < 0.05 and ***P < 0.001 compared with cells treated with Pg-LPS.

A

B

C

Figure 8

Sitagliptin attenuates the expression of inflammation-related cytokine in response to *P. gingivalis* LPS. (A) Relative levels of IL-1β, IL-6, TNF-α, iNOS, IL12-P35 and IL12-P40 mRNA determined by qPCR. (B) Proteins levels of IL-1β and (C) IL-6 in supernatants measured by ELISA. (B,C) Data were expressed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with cells treated with Pg-LPS.

Figure 9

Sitagliptin Inhibits Activation of MAPK and AKT Signaling Pathways. RAW264.7 cells were treated with or without 50 $\mu\text{g}/\text{ml}$ of sitagliptin in the absence or presence of 5 $\mu\text{g}/\text{ml}$ of Pg-LPS. (A) Cell lysates were subjected to Western blot analysis. (B) The band intensities were quantified by Image J analysis. Data were expressed as mean $\pm$ SD. * $p < 0.05$ compared with treatment with Pg-LPS. ** $p < 0.05$ compared with Group1.