Article

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rgpA-Engineered/Functionalized DNA Vaccine as a Novel Prophylactic Vaccination to Prevent *Porphyromonas gingivalis*-Induced Periodontitis: An *in Vivo* Study

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Background: Arg-gingipain A (*rgpA*) and Arg-gingipain B (*rgpB*) are crucial virulence factors associated with *Porphyromonas gingivalis* (*P. gingivalis*) and have been recognized as promising targets for antibacterial vaccines. Although vaccines containing *rgpA* have shown efficacy, the incorporation of *rgpB*, which lacks the haemagglutinin adhesin (*HA*) domain, diminishes the vaccine's effectiveness. This study aims to assess the immunogenicity of the functional *HA* domain of *rgpA* in mouse periodontitis models.

Methods: A total of 24 mice were randomly divided into four groups, each receiving different immune injections: group A received phosphate-buffered saline (PBS) as an empty control; group B received pVAX1 as a negative control (NC); group C received pVAX1-*HA*; and group D received pVAX1-*rgpA*. The mice were subjected to intramuscular injections every two weeks for a total of three administrations. Prior to each immunization, blood samples were collected for antibody detection under isoflurane anesthesia. Following the final immunization, periodontitis was induced two weeks later by using sutures soaked in a *P. gingivalis* solution. The mice were euthanized after an additional two-week period. To assess the safety of the procedure, major organs were examined through hematoxylin-eosin (HE) staining. Subsequently, the levels of IgG, IgG1, and IgG2a in the serum were quantified via enzyme-linked immunosorbent assay (ELISA). Additionally, the expression of inflammatory factors in the gingiva, including interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α), was determined using quantitative real-time reverse transcript PCR (qRT-PCR). The extent of bone loss in periodontal tissues was evaluated using micro-computed tomography (micro-CT) and HE staining.

Results: HE staining of the organs confirmed the absence of vaccine-induced toxicity *in vivo*. After the second immunization, both the *rgpA* and *HA* groups displayed significantly higher specific IgG titers in comparison to the NC and PBS groups ($p < 0.05$). Furthermore, the *rgpA* and *HA* groups exhibited a noteworthy predominance of IgG1 antibodies after three immunization doses, while there was a noticeable reduction in IgG2a levels observed following ligation with *P. gingivalis* sutures, as opposed to the NC and PBS groups ($p < 0.05$). Additionally, both the *HA* and *rgpA* groups showed a significant decrease in the expression of inflammatory factors such as IL-6, IL-1β, and TNF-α, as well as a reduction in bone loss around periodontitis-affected teeth, when compared to the NC and PBS groups ($p < 0.05$).

Conclusions: The results of this study demonstrate that the *rgpA*-engineered/functionalized *HA* gene vaccine is capable of eliciting a potent prophylactic immune response against *P. gingivalis*-induced periodontitis, effectively serving as an immunogenic and protective agent *in vivo*.

Keywords: haemagglutinin adhesin (*HA*); Arg-gingipain A (*rgpA*); *P. gingivalis*; gene vaccine; periodontitis

Introduction

Periodontitis is a complex, multifactorial oral disease characterized by the loss of supporting tissues around the teeth, ultimately resulting in tooth loss [1]. Epidemiological studies have revealed that 47% of adults aged 30 and older have periodontitis, and 64% of individuals aged 65 and above suffer from moderate to severe forms of the condition. This imposes a significant financial burden on the healthcare system [2]. *Porphyromonas gingivalis* (*P. gingivalis*), considered a low-abundance "keystone pathogen", plays a crucial role in modulating microbial symbiosis and driving the progression of periodontitis [3]. Additionally, periodontitis has epidemiological associations with various chronic disorders, including cardiovascular disease [4], Alzheimer's disease (AD) [5], and rheumatoid arthritis [6].

P. gingivalis, a gram-negative anaerobic bacterium, possesses an array of virulence factors, including cysteine proteases, lipopolysaccharide (LPS), capsular polysaccharide (CPS), and fimbriae [7]. Cysteine proteases, also known as Arg-gingipain A, B (*rgpA*, *rgpB*) and Lys-gingipain (*kgp*), are indispensable for the survival of *P. gingivalis* and play a pivotal role in the pathogen's ability to enter the endothelial system. This, in turn, induces dysbiosis by disabling host-protective antimicrobial responses [8–10]. Broad-spectrum antibiotics often fail to eradicate *P. gingivalis* and can even lead to antimicrobial resistance. In contrast, *rgpA*, *rgpB*, and *kgp* have emerged as specific virulence targets [11–13]. Several studies have suggested that immunization with pure secretory molecules can offer protection against *P. gingivalis* [14–16]. Notably, *rgpA* and *kgp* feature a haemagglutinin adhesin (*HA*) domain, which has been shown to enhance bacterial adherence to host cells and certain extracellular proteins [10,17,18]. An increasing body of evidence highlights the advantages of DNA vaccines in infectious diseases, underscoring the potential of targeting gingipains or their functional domains to prevent *P. gingivalis*-induced diseases [19].

HA comprises four putative fragments, namely Rgp44, Rgp15, Rgp17, and Rgp27, denoted as HA1, HA2, HA3, and HA4, respectively. HA1 possesses hemagglutination activity, while HA2 functions as a hemoglobin-binding receptor [20]. Some studies have targeted the HA1 antigen to induce immunity in animals, resulting in reduced alveolar bone loss in *P. gingivalis*-induced periodontitis [21,22]. Nasi Huang developed a *P. gingivalis* minor fimbriae protein by combining HA1 and HA2 in equivalent doses as adjuvants, which successfully elicited a robust specific IgG response [23]. The gene sequence of *HA* is a pivotal functional antigen with a concise sequence, which may enhance transfection efficiency. Moreover, in comparison to whole infectious pathogens or recombinant proteins, the one-step cloning of target genes into plasmid vectors offers a more convenient production method. DNA vaccines lead to protein expression *in vivo*, potentially preserving the native protein structure and essential post-translational modifications [24].

In this study, the functional *HA* domain of *rgpA* was utilized as a specific antigen to create the plasmid pVAX1-*HA*. Subsequently, the immunogenicity of this plasmid was evaluated in mouse models with *P. gingivalis*-induced periodontitis.

Materials and Methods

Bacterial Strain and Culture

Porphyromonas gingivalis (*P. gingivalis*, ATCC 33277, obtained from the American Type Culture Collection, Manassas, VA, USA) was cultured in enriched brain heart infusion (BHI) broth, which was supplemented with L-Cysteine hydrochloride anhydrous (0.4 g/L, Cat. M180920981, Macklin, Shanghai, China), yeast extract (5 g/L, Cat. 2104085-02, Oxoid, Basingstoke, Hampshire, UK), hemin (5 mg/L, Cat. 517B027, Solarbio, Beijing, China), and Vitamin K1 (5 µg/mL, Cat. H41021051, Suicheng, Qingdao, China). The culture was maintained under anaerobic conditions with a gas mixture of 10% H_2, 85% N_2, and 5% CO_2 at 37 °C [22]. The bacterial suspension was then grown to a concentration of 1×10^{10} colony-forming units per milliliter (CFU/mL) and assessed using an ultra-micro-UV spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA).

Construction of Plasmids pVAX1-HA and pVAX1-rgpA

DNA fragments encoding *rgpA* and *HA* (GenBank: NC_010729, NCBI: WP_114621325) were PCR-amplified from the genome of *P. gingivalis* ATCC 33277 using the following primers:

For *rgpA* (5.1 kb):

- Forward primer: 5′ GGGGGCTCGAGATGAGGAAATTATTATTGCTGATCGCG 3′

- Reverse primer: 5′ GGGGGTTACTTGATAGCGAGTTTCTCTACGTAAGA 3′

For *HA* (2.6 kb):

- Forward primer: 5′ CCCTCGAGATGCGCAGCGTGGATGGC 3′

- Reverse primer: 5′ GGGGTACCTTACATATCAGTTCCATCTCCC 3′

The PCR protocol followed standard conditions, including initial denaturation at 94 °C for 2 minutes, followed by 32 cycles of denaturation at 94 °C for 15 seconds, annealing at 58.2 °C for 30 seconds, and extension at 68 °C for either 5.1 minutes or 2.6 minutes, concluding with a 5-minute heating step at 68 °C [25]. Subsequently, the PCR products were separated using a 1% agarose gel and purified using the EasyPure® Quick Gel Extraction Kit (EG101-01, Trans Gen Biotech Co., Beijing, China). The DNA sequencing confirmation was performed by Genechem Co., Ltd. in Shanghai, China. Enzymatic digestion of the purified fragment products was carried out using *Xho*I (1094A, Takara Bio, Shiga, Japan) and *Kpn*I (1068S, Takara Bio, Shiga, Japan) restriction endonucleases. The resulting frag-

ments were then cloned into the eukaryotic expression vector pVAX1, which was provided along with the green fluorescence protein (GFP) gene by Gene Chem Co., Ltd. in Shanghai, China. The reconstructed plasmids were verified through DNA sequencing, and the remaining samples were stored at –20 °C until further analysis.

Cell Culture and Plasmid Transfection in Vitro and Western Blotting Survey

The 293T cell line, obtained from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (ZQ0033, ZQXZbio, Shanghai, China), was cultured in basic Dulbecco's Modified Eagle Medium (DMEM, 11965-092, Gibco, San Diego, CA, USA) supplemented with 10% fetal bovine serum (04-001-1A, BI, Kibbutz Beit Haemek, Israel). The cells were incubated at 37 °C in a 5% CO_2 atmosphere. Validation of the cell line was conducted through short tandem repeat (STR) profile analysis by Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. The absence of mycoplasma contamination was confirmed using the Mycoplasma PCR Detection Kit (ZQ505-K, ZQXZbio, Shanghai, China).

After isolation and purification using the Endo Free Plasmid Maxi Kit from Qiagen, Hilden, Germany, the plasmids (1 μg/μL) were transfected into 293T cells (1.5×10^5 cells/well) using 3 μg/well of Lipofectamine TM 2000 (2028090, Invitrogen, Carlsbad, CA, USA) for 48 hours. The cells were then lysed with 100 μL of radio immunoprecipitation assay (RIPA) buffer (150 mM, R0020, Solar Bio, Beijing, China) at 4 °C, quantified using a bicinchoninic acid (BCA) protein assay kit (PC0020, Solar Bio, Beijing, China), and identified using western blotting. Proteins (35 μg/well) were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), which included a 10% separation polyacrylamide gel and 4% polyacrylamide concentration gel (P0014A, Beyotime, Shanghai, China). Electrophoresis was initiated at 60 V to load the samples into the separation gel, and then the voltage was adjusted to 160 V to continue electrophoresis until the bromophenol blue dye ran out. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (PVDF, IPVH00010, Millipore, Billerica, MA, USA) using an electrophoretic transfer system (1703930, Bio-Rad, Hercules, CA, USA). The PVDF membranes were then blocked with 5% skim milk powder in Tris Buffered saline Tween (TBST) (ST671, Beyotime, Shanghai, China) for 1 hour. Subsequently, the membranes were incubated with anti-GFP polyclonal rabbit IgG (1:1000, c0619, Santa Cruz Biotechnology, CA, USA) overnight at 4 °C and matched with horse radish peroxidase (HRP)-conjugated anti-rabbit antibodies (1:5000, D0219, Santa Cruz Biotechnology, CA, USA) at room temperature for 1 hour. Finally, enhanced chemiluminescence substrates (1705062, Bio-Rad, Hercules, CA, USA) were added to the film's surface to capture images using the Amersham Imager 600 (Cytiva, UT, USA).

Preventive Immunization and P. gingivalis-Induced Periodontitis Model Construction

Female C57 mice, aged 5–7 weeks and weighing 17–20 g, were procured from Sibeifu Biotechnology Co., Ltd. (Beijing, China). The mice were randomly divided into four groups (n = 6) based on different injection substances, following the random number method. These groups were named phosphate-buffered saline (PBS), pVAX1 (negative control, NC), pVAX1-*HA* (*HA*), and pVAX1-*rgpA* (*rgpA*). On days 0, 14, and 28, the mice were intramuscularly immunized using a micro-syringe with the following substances:

- PBS/100 μL
- pVAX1/100 μg
- pVAX1-*HA*/100 μg
- pVAX1-*rgpA*/100 μg

This injection was administered along with 0.125% bupivacaine hydrochloride (R014301, RHAWN, Shanghai, China) after the mice were anesthetized using isoflurane (R510-22-10, RWD, Shenzhen, China). A week after the last immunization, silk thread soaked in a *P. gingivalis* suspension (approximately 1×10^{10} CFU/mL) was ligated around the second maxillary molar for a period of 2 weeks to induce periodontitis. It has been established that alveolar bone loss in periodontitis models primarily occurs in the early stage (3–9 days) and decreases in the later stage (9–18 days) [26]. Bone loss was assessed by measuring the distance between the cemento-enamel junction and alveolar bone crest (CEJ-ABC) [27]. Blood samples were collected from the posterior ocular venous plexus after anesthesia before each immunization and after the induction of periodontitis. These blood samples were then subjected to centrifugation at 3000 rpm for a duration of 20 minutes to obtain serum, which was subsequently stored at –20 °C until further analysis. Finally, all animals were euthanized through excessive inhalation of isoflurane. It is important to note that all animal experiments were approved by the Ethics Committee of the Stomatology School of Shandong University (Approval No. 20190102).

Histological Observation and Micro-Computed Tomography (Micro-CT) Analysis

Twelve days after ligation, the maxilla, heart, liver, spleen, kidney, and lung of all the subjects were collected and preserved in a 4% paraformaldehyde solution (P0099, Beyotime, Shanghai, China) for a period of two days. Subsequently, half of the specimens underwent hematoxylin-eosin (HE) staining and were examined under a Leica DM-RBEs microscope (DMR, Leica, Wetzlar, Germany). The analysis was conducted using Image J software (version 1.53t, National Institute of Health, Bethesda, MD, USA).

The maxillary bone was imaged using the Inveon MM computed tomography (CT) Acquisition Workplace (SIEMENS, Munich, Germany) at 80 kV and 500 μA, with an effective pixel size of 8.89 μm, a scanning time of 965 seconds, a 360° rotation angle, and an exposure time of

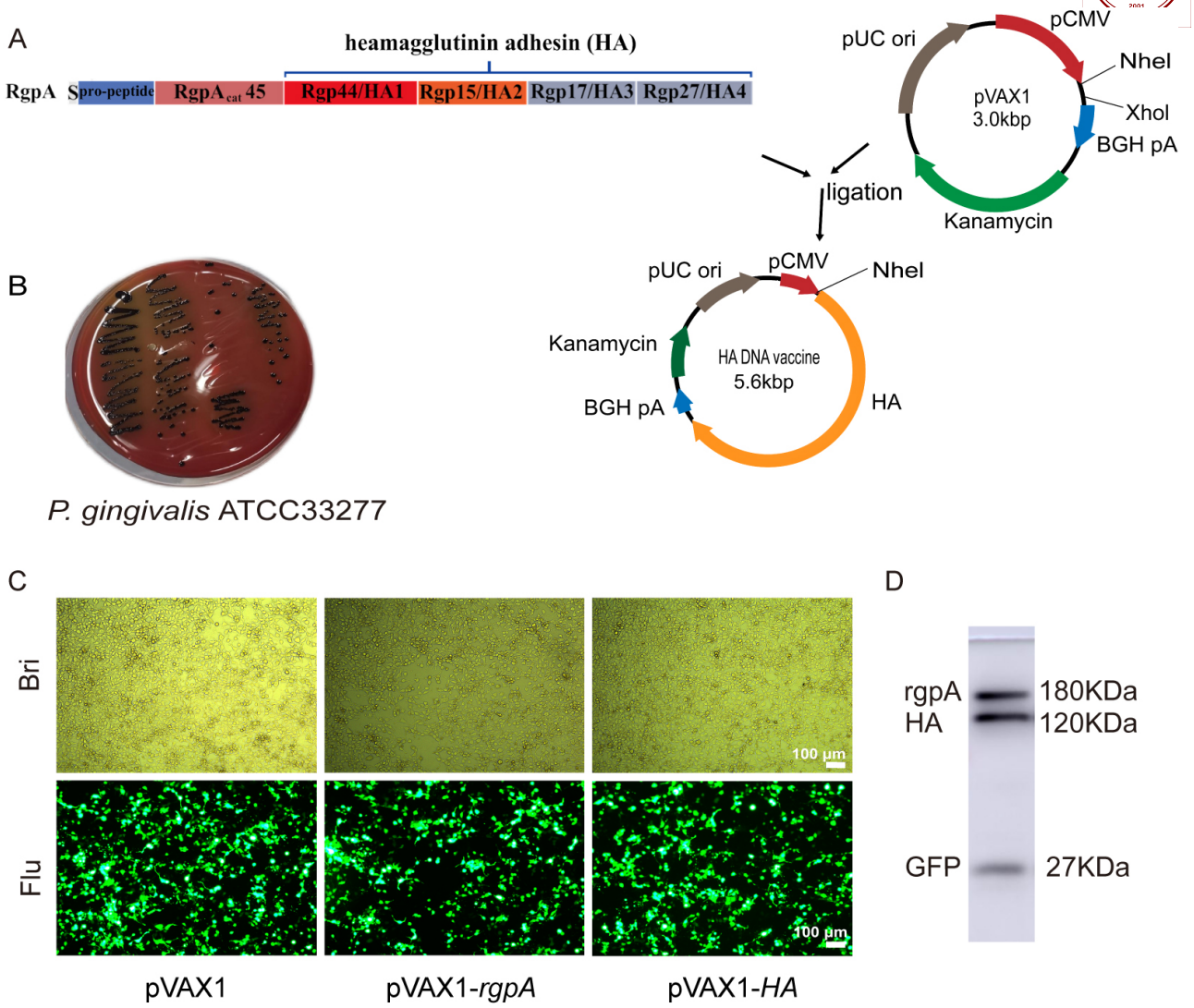

Fig. 1. Plasmid construction and cultivation of *Porphyromonas gingivalis* (*P. gingivalis*). (A) The relation of haemagglutinin adhesin (*HA*) in Arg-gingipain A (*rgpA*) and the vector and plasmid map before and after construction. (B) Culture of *P. gingivalis* (ATCC 33277). (C) Recombinant plasmids were transfected into 293T cells and were observed under a fluorescence microscope. (D) Western blotting analysis of the gene expression. The marker was obtained from Thermo Fisher Scientific (Waltham, MA, USA). GFP, green fluorescence protein.

2000 ms. The captured images were then reconstructed in three dimensions (3D) using COBRA_Exxim (EXXIM Computing Corp., Livermore, CA, USA). Vertical bone loss was quantified by measuring the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) on the distal and mesial sides of the second molar on each buccal surface using on-screen measurements of Inveon Research Workplace (SIEMENS, Munich, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum IgG levels were detected using ELISA, following established procedures, which also included assessing IgG subclasses IgG1 and IgG2a [25]. Here's a brief overview: Initially, 10 μg of the *P. gingivalis* extraction suspension was coated onto 96-well plates (Corning Laboratories, NYC, NY, USA) and blocked for 24 hours at 4 °C. The plates were then washed three times and blocked using 3% bovine serum (04-001-1A, BI, Israel) in PBS (C0221A, Beyotime, Shanghai, China) for 1 hour. Following additional washing steps, gradient dilution samples were incubated at 37 °C for 60 minutes. Subsequently, the plates were incubated with HRP-conjugated Goat anti-mouse IgG (1:1000, ab150113, Abcam, Cambridge, UK), Rabbit anti-mouse IgG1 (1:1000, ab125913, Abcam, Cambridge, UK), and Rabbit anti-mouse IgG2a (1:1000, ab190463, Abcam, Cambridge, UK). The absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The titers were calculated by determining the reciprocal $\log_{10}$ value of the dilution.

Table 1. Inflammatory factor primer sequence.

	Forward primer	Reverse primer
IL-1β	5′GAAATGCCACCTTTTGACAGTG3′	5′TGGATGCTCTCATCAGGACAG3′
IL-6	5′CTCTGGCTTTGTCTTTCTTGTTATCTTT3′	5′AGTTGTGCAATGGCAATTCTGA3′
TNF-α	5′AGG CGGTGCCTATGTCTCAG3′	5′GCTCCTCCA CTTGGTGGTTT3′
GAPDH	5′CTGCCCAGA ACATCATCCCT3′	5′TGGTCCTCAGTGTAGC CCAAG3′

IL-6, interleukin-6; *IL-1β*, interleukin-1β; *TNF-α*, tumor necrosis factor alpha; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Quantitative Real Time Reverse Transcript PCR (qRT-PCR)

In this study, the mRNA expression levels of interleukin-6 (*IL-6*), interleukin-1β (*IL-1β*), and tumor necrosis factor alpha (*TNF-α*) in the gingivae were detected by qRT-PCR. The summary of the methods used for this analysis are provided:

(1) Total RNA Extraction:

Total RNA was extracted from mouse periodontal gingivae using the FastPure tissue total RNA Isolation Kit V2 (RC112-01, Vazyme, Nanjing, China).

(2) Reverse Transcription:

Reverse transcription of RNA to cDNA was carried out using the PrimeScript RT reagent Kit with gDNA Eraser (RR047A, Takara Bio, Shiga, Japan).

(3) qRT-PCR:

Quantitative real-time PCR was performed using SYBR Green qPCR Mix (AH0101-A, Spark Jade, Jinan, China) following the manufacturer's protocol.

Each PCR reaction included 10 µL of 2 × SYBR qPCR Mix, 2 µL of cDNA (20 ng), 0.4 µL of forward and reverse primers (10 µM each), and RNase-free H_2O was added to reach a final volume of 20 µL. The reactions were run on a Light Cycler 480 Real-Time PCR System (05015243001, Roche, Basel, Switzerland).

PCR amplification conditions involved an initial step of 2–4 minutes at 94 °C, followed by 40 cycles of 10 seconds at 94 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data, with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

The specific primer sequences used in the qRT-PCR analysis can be found in Table 1.

Statistical Analysis

The data analysis for this study was carried out using GraphPad Prism software (version 6.0.0, GraphPad Software Inc., Boston, CA, USA). To assess the differences between groups, the following statistical methods were employed: One-Way Analysis of Variance (ANOVA): ANOVA is a statistical test used to analyze variance between multiple groups. Subsequently, an Unpaired *T*-Test: The unpaired *t*-test is used to compare means between two independent groups.

The results were presented as mean ± standard deviation (SD) in the graphs, and statistical significance was determined at a significance level of $p < 0.05$. When *p*-values were less than 0.05, differences were considered statistically significant.

Results

Plasmids Construction and Expression

Fig. 1A displays the relationships between domain gene positions and plasmid profiles. *Porphyromonas gingivalis* (*P. gingivalis*, ATCC 33277) was cultivated on BHI blood agar under anaerobic conditions for 5–7 days until colonies with black pigmentation were visible (Fig. 1B). The expression of green fluorescent protein (GFP) in 293T cells after plasmid transfection was observed under an inverted fluorescence microscope (Fig. 1C). To assess protein expression in the cytoplasm of *HA*/*rgpA*-transfected 293T cells, Western blotting was conducted after incubation with anti-GFP antibodies (Fig. 1D). These findings confirmed the successful construction of plasmids capable of expressing recombinant HA and rgpA proteins in cells.

Enhancement of IgG and IgG1 Responses in Periodontitis Mice after Immunization

The experimental procedure is depicted in Fig. 2A, and the establishment of the periodontitis model through silk ligation is presented in Fig. 2B. The quantification of *P. gingivalis*-specific antibody production in serum was performed using the enzyme-linked immunosorbent assay (ELISA) method. After intramuscular immunization with pVAX1-*HA* and pVAX1-*rgpA*, a significant elevation in IgG levels was observed at weeks 2, 4, and 6 in comparison to the pVAX1 (negative control) and PBS groups ($p < 0.05$), as demonstrated in Fig. 2C. The difference was particularly pronounced at week 4, after which it stabilized or slightly declined. Nevertheless, there was no significant distinction between the pVAX1-*HA* and pVAX1-*rgpA* vaccination groups ($p > 0.05$) (Fig. 2D).

Concurrently with the increase in IgG secretion, the level of IgG1 substantially rose in the pVAX1-*HA* and pVAX1-*rgpA* groups, reaching its peak at week 4 ($p < 0.05$), in comparison to immunization with the negative control (NC) and PBS. In contrast, IgG2a displayed no significant alteration ($p > 0.05$) (Fig. 2E). This elicited a Th2-biased response, with an IgG1/IgG2a ratio greater than 1. Subsequent to *P. gingivalis* infection, the IgG2a response increased in all groups, particularly in the control groups

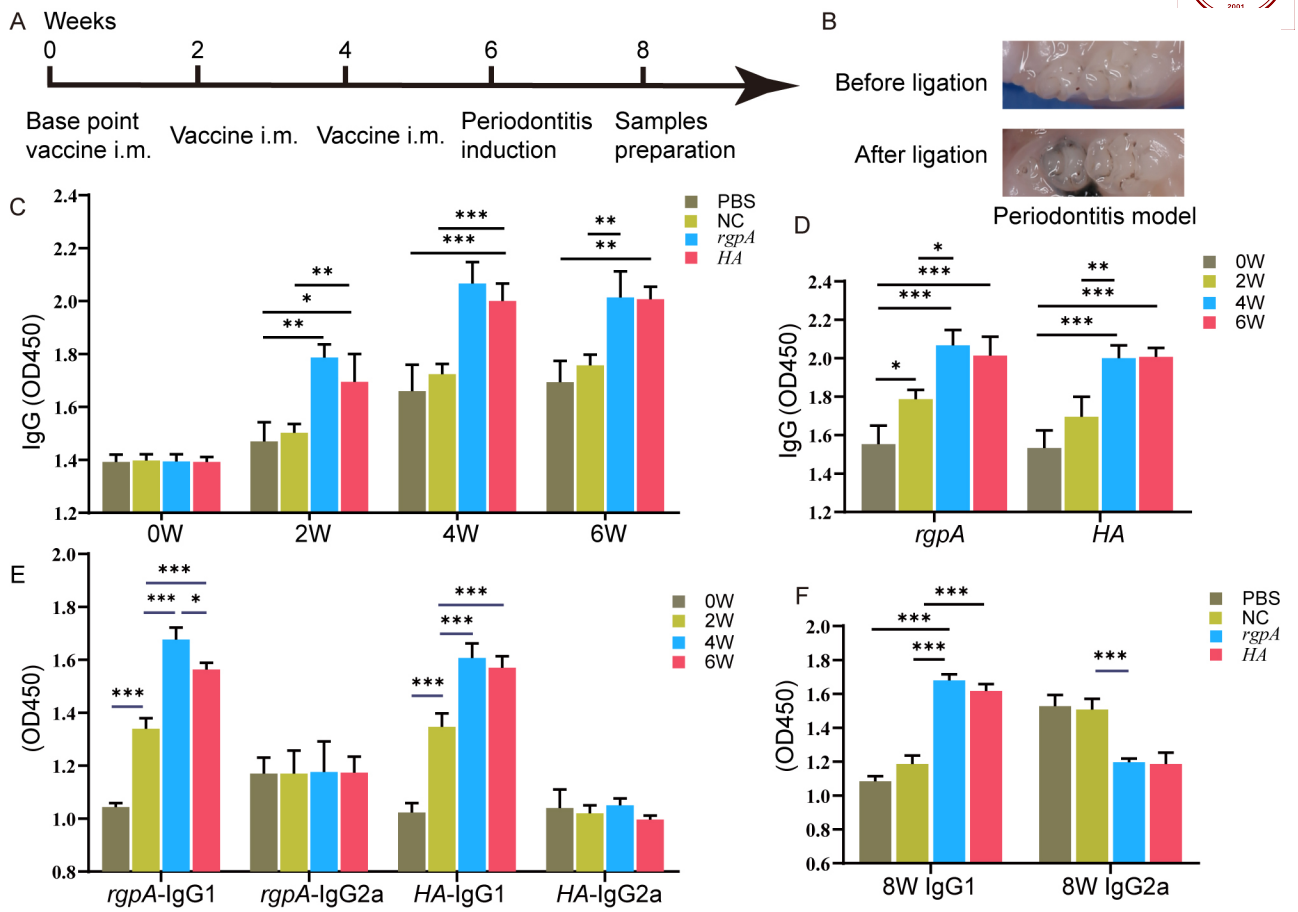

Fig. 2. IgG and subclass antibody response in serum of periodontitis mice after immunization. (A) Experiment chart flow. (B) Construction of a mouse model of periodontitis. (C) Serum IgG levels at 0, 2, 4, and 6 weeks. (D) Longitudinal comparison of IgG in groups pVAX1-*rgpA* and pVAX1-*HA*. (E) A predominant IgG1 response was observed in the *rgpA* and *HA* groups post-immunization, whereas IgG2a did not change significantly. (F) IgG2a levels increased significantly in control groups post-challenge, whereas IgG1 remained relatively high in the *HA* and *rgpA* groups. Comparisons were performed using one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. PBS, phosphate-buffered saline; NC, negtive control; ANOVA, One-Way Analysis of Variance; OD, optical density.

compared to the *HA* and *rgpA* groups ($p < 0.05$), while IgG1 remained relatively high (Fig. 2F). In summary, immunization with pVAX1-*rgpA* and pVAX1-*HA* induces a more robust humoral immune response by promoting the increase of Th2-biased IgG1, thereby achieving immune prevention.

Histological Observation and Micro-Computed Tomography (Micro-CT) Analysis of Ligated Teeth and Periodontal Tissue

After two weeks of ligation of molars with silk threads soaked in a *P. gingivalis* suspension, bone loss was assessed by measuring the distance between the cemento-enamel junction and alveolar bone crest (CEJ-ABC) at the distal and mesial aspects of the second molar, as indicated by the red line in Fig. 3A. HE staining of the teeth in the subjects revealed bone loss and gingival inflammation in all the groups. The gingival sulcus epithelium around the ligature teeth exhibited varying degrees of erosion or ulceration in all the test groups, as marked by the green line indicating the distance of CEJ-ABC (Fig. 3B). The results obtained from micro-CT indicated a significant reduction in periodontal bone loss in the *rgpA* (ABC-CEJ, 0.24 $\pm$ 0.02 mm) and HA (0.22 $\pm$ 0.02 mm) groups when compared to the NC and PBS groups ($p < 0.05$), with no significant difference observed between the *HA* and *rgpA* groups ($p > 0.05$) (Fig. 3C). In summary, these findings suggest that the *rgpA* and *HA* DNA vaccines exhibit similar efficacy in preventing *P. gingivalis*-induced periodontal bone loss.

qRT-PCR Assay of Inflammatory Factors in Gingival Tissues

Two weeks after the establishment of the periodontitis model, the maxillary bone tissue on one side of the mouse was collected immediately following euthanasia (Fig. 4A), frozen at –80 °C, and subjected to qRT-PCR to assess the expression of inflammatory factors in the gingival tissue within the periodontitis area. Gene expression of the relevant inflammatory factors, including IL-1β, TNF-α, and IL-6, exhibited a significant decrease in the *HA* and *rgpA* immunization groups compared to the NC and PBS control

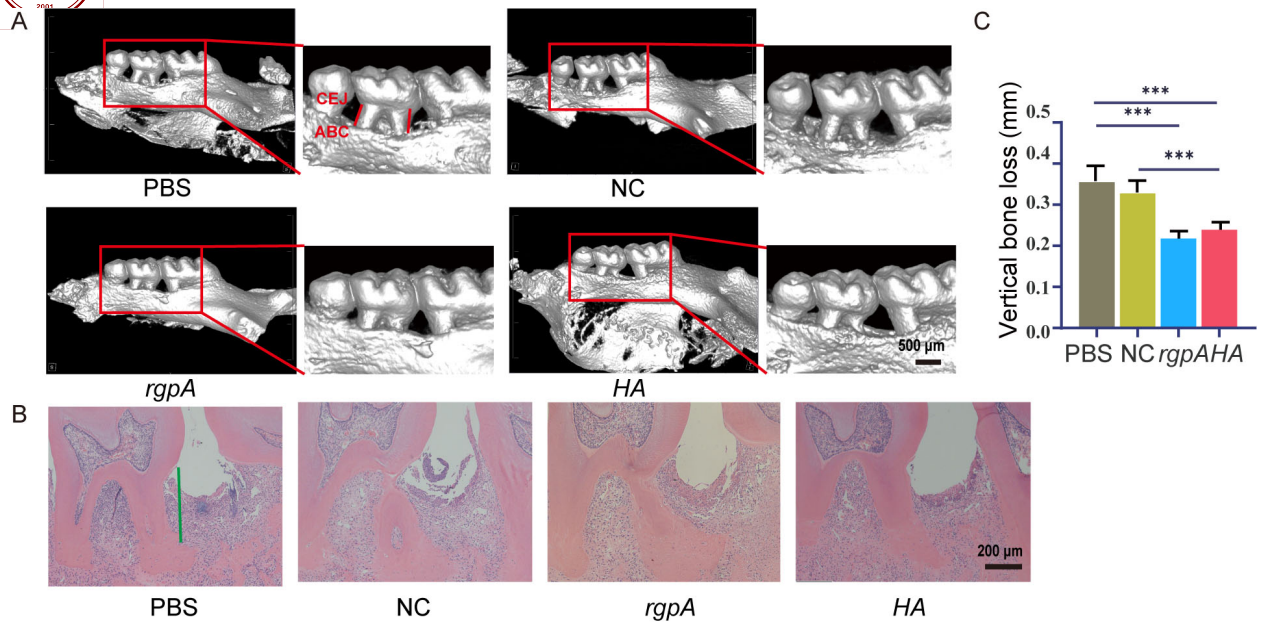

Fig. 3. Histological observation and micro-computed tomography (CT) analysis of periodontal tissue. (A) Vertical bone loss was assessed by measuring the distance between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) at the distal and the mesial of the second molar. (B) Gingival inflammation of subject teeth after hematoxylin-eosin (HE) staining. The green line illustrates the distance between CEJ and ABC on the slice. (C) Quantitative analysis of alveolar bone absorption. Values are represented as mean $\pm$ standard deviation (SD). ***$p < 0.001$.

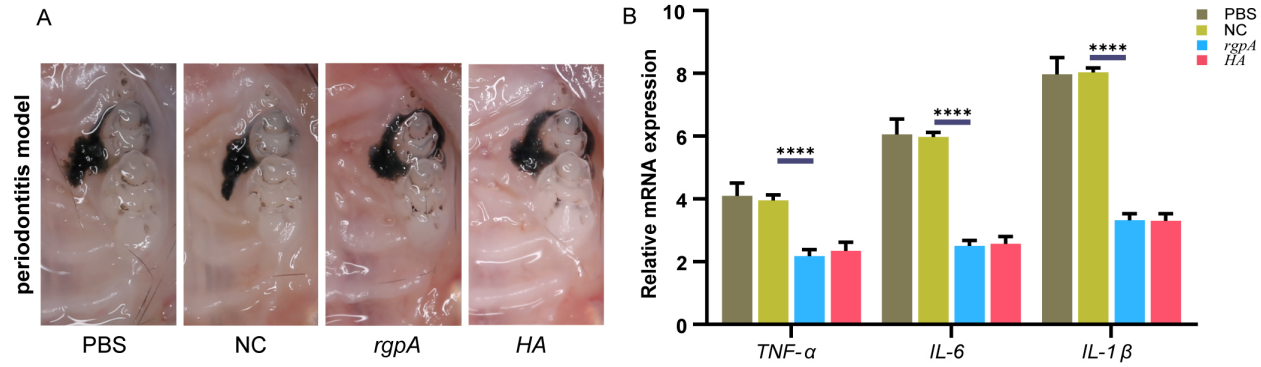

Fig. 4. Expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in periodontal gingivae tissues. (A) The maxillary bone tissue of one side of the mouse in four groups. (B) qRT-PCR analysis of *TNF-α*, *IL-6*, and *IL-1β* mRNA in periodontal gingivae. The $2^{-\Delta\Delta Ct}$ method was utilized to analyze data normalized to GAPDH expression. ****$p < 0.0001$.

groups (Fig. 4B) ($p < 0.05$). In conclusion, these findings support the notion that the prophylactic administration of pVAX1-*HA* and pVAX1-*rgpA* gene vaccines effectively reduces the expression of local inflammatory factors in periodontitis, thus offering potential as a preventive measure against alveolar bone loss.

Histological Study of Organs for Biosafety

To assess the safety of plasmid injection, the hearts, livers, spleens, lungs, and kidneys of mice from each group were collected and subjected to HE staining. The results reveal no significant abnormalities in the organs of the mice treated with genetic vaccines compared to those of the normal blank control mice (Fig. 5).

Discussion

Periodontal disease affects a substantial portion of the adult population worldwide, underscoring the urgency of developing an effective antigen vaccine for prevention and treatment. Immunobiotherapy presents a promising strategy for targeting specific pathogens. Previous research has explored various approaches, including the purification of molecules from organisms, and the use of recombinant proteins or molecules, yielding varying degrees of success [26,28,29].

DNA vaccines introduce a novel approach to managing infectious diseases and offer potential advantages over traditional protein-based vaccines [30,31]. In order to max-

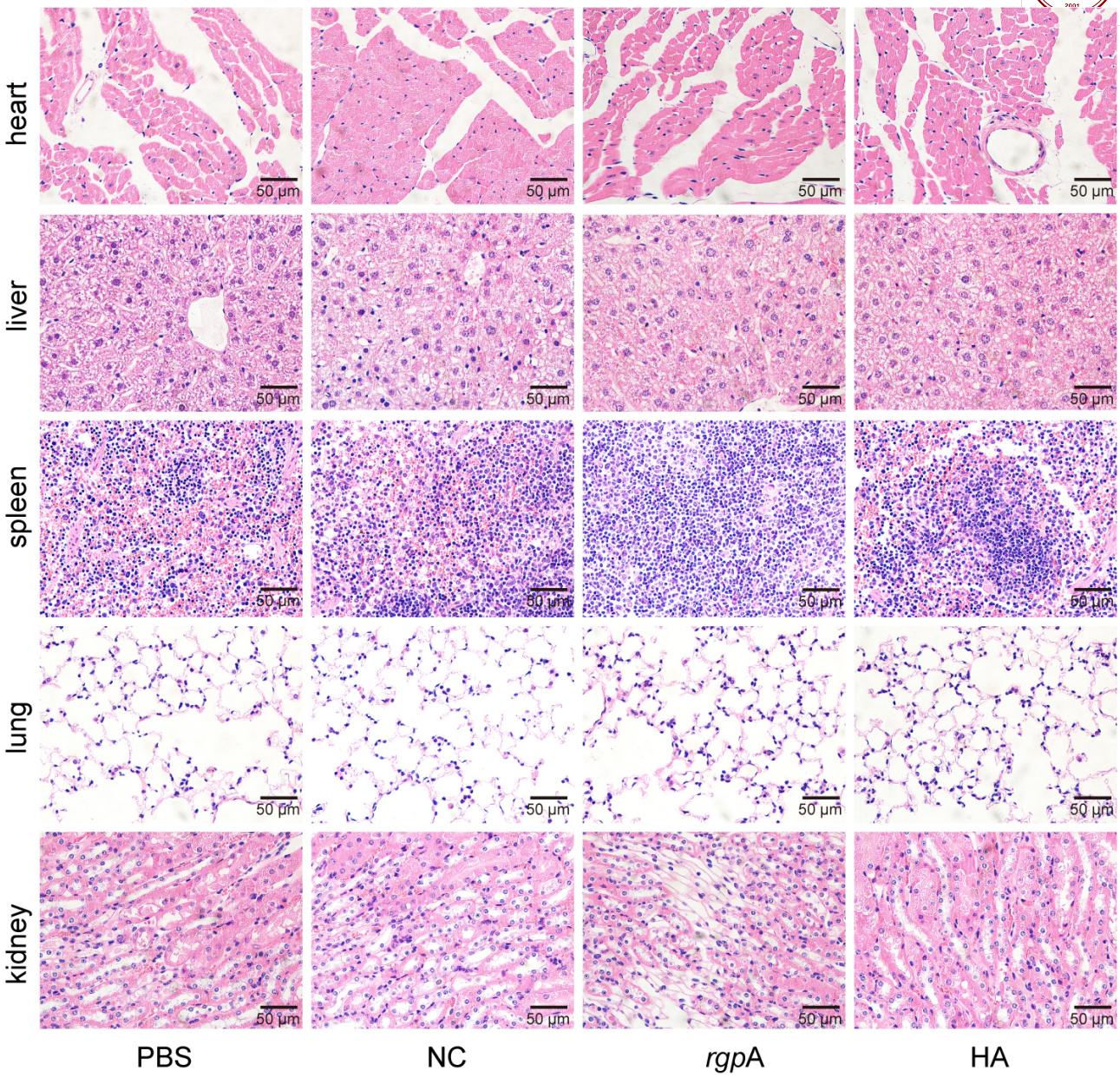

Fig. 5. Biosafety was assessed by HE staining of major organs. Scale bar: 50 μm.

imize the efficacy of DNA vaccines, the identification of specific antigen genes becomes paramount. Several studies have focused on the external secretion virulence factors of *Porphyromonas gingivalis* (*P. gingivalis*), including minor and major fimbriae, Arg-gingipain, and Lys-gingipain antigens (*rgpA*, *kgp*), as well as the haemagglutinin adhesin (*HA*) of *rgpA* and *kgp*, with the aim of eliciting a protective immune response [23,32,33]. This research concentrates on the *HA* and *rgpA* antigen genes for vaccine immunization, with particular emphasis on *HA* as a novel gene vaccine.

Our study demonstrates that both the *HA* and *rgpA* genes can significantly enhance IgG production and effectively prevent *P. gingivalis*-induced alveolar bone loss. These findings are consistent with previous research, such as the study by Gibson FC *et al.* [28], which reported that immunization with the RgpA antigen could protect against *P. gingivalis*-induced bone loss. Additionally, studies by O'Brien-Simpson *et al.* [15,34] have also shown similar outcomes, where immunizing mice with RgpA-Kgp HA peptides led to an IgG response and triggered a protective immune response. Notably, *P. gingivalis*-induced platelet aggregation in plasma is linked to the HA1 domain, rather than the Rgp proteinase [35]. A DNA vaccine expressing HA1 has proven effective in inducing a protective response [21,22]. Furthermore, the HA2 domain, serving as a major high-affinity receptor, plays a critical role in hemoglobin binding and heme capture, which are essential for *P. gingivalis* growth [35]. Nevertheless, the utilization of these two fragments in genetic vaccines remains unexplored. Our current study has unveiled that both *HA* and *rgpA* DNA vaccines exhibit comparable efficacy in eliciting IgG responses. Notably, Schommer *et al.* [36] utilized chondroiti-

nase ABC (Cho ABC) to deliver intramuscular injections of plasmid DNA, which resulted in the degradation of the chondroitin sulfate scaffolding barrier. This approach facilitated the dispersion and activation of genes within tissues, thereby enhancing the synthesis of functional IgG in the organism.

IgG1 is known to promote a Th2-type immune response and displays a noticeable increase both before and after bacterial infection, signifying the initiation of a protective response [37]. In our current study, we observed a transition of the humoral response towards an anti-inflammatory profile, marked by elevated serum IgG1 levels and reduced serum IgG2a levels. These findings are in concordance with prior research, which illustrated that immunization with recombinant RgpA or RgpA-Kgp can lead to a shift in the humoral response characterized by increased IgG1 [16,38].

Conversely, in the non-vaccine group, *P. gingivalis* infection triggered a predominant IgG2a antibody response [39]. Zhu *et al*. [40] also reported significantly higher serum IgG levels following HA2 immunization in rats. Moreover, Miyachi *et al*. [41] demonstrated that the gene vaccine *rgpA* could induce both IgG1 and IgG2b antibodies through Th2 responses. It's worth noting that the presence of pathogen-specific afucosylated IgG1 responses can have beneficial effects, as seen in the protection against malaria and HIV [42,43]. Julie Van Coillie *et al*. [44] showed that BNT162b2 mRNA vaccination induces transient afucosylated anti-S IgG1 responses in immunologically naive individuals. Nevertheless, further investigations are needed to clarify the specific clinical scenarios in which robust afucosylated responses would be advantageous. These results suggest that additional comprehensive research is required to delve into the antibody response generated by the DNA vaccine in our study.

Inflammatory factors (IL-6, IL-1β, TNF-α) were also significantly reduced in the inflamed gingival tissue following prophylactic immunization, mirroring the antibody response. In our study, *HA* DNA immunization prevented 30% of alveolar bone loss in mice with periodontitis, showing results similar to *rgpA* DNA immunization. This suggests that both *rgpA* and *HA* gene vaccines exhibit high immunogenicity and are immunodominant. These findings align with previous research, highlighting the potential of *HA* as a highly immunogenic antigen, particularly when shorter, targeted DNA sequences are employed to improve transfection efficiency and immunogenicity.

To enhance the antigen-specific immune response, the inclusion of vaccine adjuvants has proven to be an effective strategy for stimulating the innate immune response. It's important to note that nearly all authorized vaccines incorporate adjuvants, underscoring the significance of optimizing vaccine formulations, particularly those involving adjuvants, in the context of clinical vaccine development.

Conclusions

In conclusion, this study provides evidence that immunization with pVAX1-*HA* and pVAX1-*rgpA* induces prophylactic anti-inflammatory immunity. It accomplishes this by significantly increasing serum IgG and IgG1 levels while reducing *IL-6*, *IL-1*β, and *TNF*-α. This immune response effectively prevents alveolar bone loss in *P. gingivalis*-induced periodontitis. These DNA vaccines hold promise for potential application in adjunctive treatments for periodontitis patients in combination with scaling and root planning. Additionally, they may have a role in preventing systemic diseases exacerbated by *P. gingivalis* infections.

Availability of Data and Materials

The original data mentioned in the study are included in the article, and more information can be requested from the corresponding authors.

Author Contributions

XF, JL and SZ designed the research study. XF, XL and HR performed the research. XF, JL and SZ analyzed the data. XF and HR prepared the figures. The article was drafted by XF with input from JL and SZ. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Informed consent was obtained from all subjects involved in the study. All animal experiments were approved by the Ethics Committee of the Stomatology School of Shandong University (Approval No. 20190102). This study conformed the ARRIVE guidelines.

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Conflict of Interest

The authors declare no conflict of interest.

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