Pharmacodynamics of siRNARANKL and Oestrogen Loaded MSNs-CADY on Human Periodontal Ligament Stem Cells with Porphyromonas gingivalis infection

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Pharmacodynamics of siRNA_{RANKL} and Oestrogen Loaded MSNs-CADY on Human Periodontal Ligament Stem Cells with *Porphyromonas gingivalis* infection

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Abstract

Background: Periodontitis irreversibly invades and destroys periodontal supporting tissues, loses the ability of periodontal regeneration and restoration, and eventually leads to tooth loosening and loss. periodontal ligament stem cells (PDLSCs) hold great promises for periodontal tissue regeneration which was the potential target of periodontitis treatment, siRNA_{RANKL} and oestrogen can help PDLSCs maintain normal function, however, it was very difficult for siRNA_{RANKL} and oestrogen to get into PDLSCs. Here, Cell penetrating peptide CADY was modified on the surface of siRNA_{RANKL} and oestrogen loaded mesoporous silica nanoparticles (MSNs) to carry them into *Porphyromonas gingivalis* infected PDLSCs, Then further affect the proliferation of PDLSCs.

Methods: 120-150 nm Mesoporous silica nanoparticles (MSNs) was prepared, and the biocompatibility, loading capacity and drug release property were tested; MSNs was modified by penetrating peptide CADY and the prepared MSNs/CADY was loaded with siRNA_{RANKL} and oestrogen; In vitro drug release of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY was tested by using semi-permeable dialysis bag diffusion; Cellular uptake and internalization of FITC-Labeled MSNs and FITC-Labeled MSNs-CADY was observed by use of Laser confocal microscopy; Finally, the effect of siRNA_{RANKL} and oestrogen loaded MSNs-CADY on cell proliferation of *Porphyromonas gingivalis* infected

human periodontal ligament stem cells was tested by MTT assay.

Results: according to the results, MSNs-CADY with a concentration of 6.25-200 ug/mL have no toxic to PDLSCs; 24.6 mg oestrogen and 0.5 mM siRNA_{RANKL} can be loaded into 1mg of MSNs-CADY; and drug loaded MSNs-CADY nanodrug carriers can release siRNA_{RANKL} and oestrogen stably for at least 48 h; After modification with cell penetrating peptide CADY, more MSNs-CADY can be taken by PDLSCs. siRNA_{RANKL}/oestrogen/MSNs-CADY can increase the proliferation of PDLSCs significantly.

Conclusion: siRNA_{RANKL}/oestrogen/MSNs-CADY constructed can significantly improve the cell proliferation of *P-gingivalis* infected PDLSCs, this nano drug carrier has the potential to be used in PDLSCs -based periodontitis treatment, this work provided a useful theoretical basis and therapeutic ideas for the treatment of periodontitis.

Keywords

Periodontitis, PDLSCs, RANKL, Oestrogen, MSNs,

Background

Periodontitis is a chronic inflammatory disease of periodontal supporting tissues. It mainly relies on local immune response. It irreversibly invades and destroys periodontal supporting tissues, loses the ability of

periodontal regeneration and restoration, and eventually leads to tooth loosening and loss, it is an inflammatory disease negatively affecting up to 15% of adults worldwide [1]. Plaque bacteria and their metabolites are the initiating factors of periodontal disease. *Porphyromonas gingivalis* (*P.gingivalis*) is one of the most virulent periodontal pathogens. It is closely related to the severity and recurrence of periodontal lesions [2]. It is generally agreed that *P.gingivalis* infection effect bone formation and bone resorption and then effect the reconstruction of the periodontal ligament (PDL) [3]. Recent studies reported that periodontal ligament stem cells (PDLSCs) hold great promises for periodontal tissue regeneration, PDLSCs possess the capacity for self-renewal and multipotent differentiation, and are able to form cementum/PDL-like tissue in vivo [4, 5].

As we know, the Receptor activator of NF- κ B ligand/osteoprotegerin (RANKL/OPG) system plays a very important role in the regulation of osteoclastogenesis in bone systems [6], Lipopolysaccharide (LPS) is the most important pathogenic factor of *P.gingivalis*, the main pathogen of periodontitis. It can promote the up-regulation of lymphocyte inflammatory factor secretion, activate osteoclast expression, and lead to alveolar bone destruction and absorption [7]. It has been reported that LPS of *P.gingivalis* can up-regulate the expression of RANKL in periodontal ligament cells and osteoblasts [8]. Oestrogen (E2) is a

hormone that affecting biological processes that include bone formation, angiogenesis and autoimmunity [9-11]. It was also reported that exogenous E2 enhanced the proliferation and osteogenic differentiation of hPDLSCs during in vitro culture [12]. Theoretically, the expression of RANKL should be decreased in hPDLSCs which infected by *P.gingivalis*, and exogenous E2 at an appropriate concentration can also help hPDLSCs maintain normal function. Therefore, drug carrier with high drug loading capacity and good release performance was needed.

The main purpose of this study was to construct a mesoporous nano drug carrier which can improve the internalization efficiency of siRNA_{RANKL} and oestrogen, and then to evaluate the pharmacodynamics of the siRNA_{RANKL} and oestrogen loaded drug carrier on proliferation of *P-gingivalis* infected PDLSCs.our study provided a useful theoretical basis and therapeutic ideas for the treatment of periodontitis.

Methods

Construction of CADY modified MSNs

Firstly, Mesoporous silica nanoparticles with an diameter of about 120 nm were prepared according to Liu's method with some modification [13]. In brief, 5 mL $\text{NH}_3 \cdot \text{H}_2\text{O}$, 5 mL ddH₂O and 50 mL ethanol were mixed in and flaked, then the mixed solution was heated to 60 °C. and then 2 mL tetraethoxysilane (TEOS) was added drop wise at a speed of 0.5 mL/min after diluted in ethanol. Subsequently, the solution was heated at 60 °C

under continuous stirring for 4 h. finally, then unmodified mesoporous silica nanoparticles from the reaction was collected by centrifugation at 12000g and washed 3 times with ethanol, and dried at 60 °C overnight. Secondly, 1 mL of the carboxy-modified (3-aminopropyl) triethoxysilane (APTES) reacted with 3 mL of succinic anhydride (1mg/mL) in the presence of dichloromethane at room temperature for 24 h, then 5 mL carboxy-modified APTES was added to 10 mg MSNs in ethanol and stirring for 24 h. Thereafter, 10 mg MSNs-COOH were separated and washed with water for several times. FITC-APTES was prepared by added 200 uL FITC (1 mg/mL) into 5 mL APTES in the presence of methanol under dark and dry condition. Then the 1mL FITC-APTES was added to 10 mg MSNs-COOH and stirring for 24 h. Finally, 100 mg of methoxy polyethylene glycol succinimidyl carbonate (mPEG-SC) was dissolved in 25 mL of dimethyl sulfoxide (DMSO), and 20 mL of this DMSO solution was added to the 10 mg FITC-labeled MSNs, followed by stirring at 4 °C for 24 h.

Thirdly, CADY peptide (GLWWKAWWKAWWKSLWWRKRKRKA) was purchased from Sangon Biotech Co. (Shang hai; HPLC-purified; purity >95%, identified by MS). Cross-linking reagents EDC and NHS was used to covalently conjugated CADY was onto MSNs, 3 mg of MSNs-COOH was dissolved in 1 ml of MES buffer (pH 6.0), then 6 mg of EDC and 6.4 mg of NHS was added and the mixture was stirred at

4 °C for 6 h. Then the solution was washed for times with additional ddH₂O. Then 100 µl of CADY solution (10 mg/ml) was added and the mixture was stirred for another 4 hours at 4 °C, washed three times in PBS.

siRNA_{RANKL} and oestrogen Loaded

About 15 mg of MSNs-CADY was dispersed into the solution with siRNA_{RANKL} (50 µg/mL) and oestrogen (1mg/mL) under at 4 °C for 48 h. The siRNA_{RANKL}/oestrogen/MSNs-CADY were centrifuged, washed with PBS for three times, and then dried under vacuum at room temperature overnight.

In Vitro Drug Release of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY

In vitro release of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY was evaluated by using semi-permeable dialysis bag diffusion. 10 mg of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY were dispersed in 5 mL of PBS (pH 7.4) separately with stirring and ultrasonication. Then, release mediums were placed in semipermeable dialysis bags and immersed into 15 mL, respectively, at 37 °C with gentle shaking (120 rpm). At different time intervals, 4 mL of the PBS from semipermeable dialysis bags was withdrawn to measure the amount of released oestrogen by human estradiol (E2) ELISA kit purchased from mibio (mlbio, Hangzhou, china,

www.mlbio.cn), and the release of siRNA_{RANKL} was test by use of nanodrop (ThermoFisher). An equal amount (4 mL) of fresh medium was added to the semipermeable dialysis bags after taking a measurement.

PDLSCs Cell culture

PDLSCs cells were purchased from Saibaokang (Shanghai) Biotechnology Co., Ltd. (<http://www.icellbioscience.com>, shanghai, china) and cultured in the media with α -MEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS, 2mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere of 5% CO₂.

Cellular Internalization

The PDLSCs cells were seeded on 60 mm dishes (Corning Incorporated). 100 mg/mL FITC-labeled MSNs and FITC-labeled MSNs-CADY was added to the cells, cells were washed with PBS four times after incubated for 12 h, then stained with LysoTracker Red DND-99 (Beyotime Institute of Biotechnology, Haimen, China) for 30 min, washed with PBS 3 times, added 2mL of 4% paraformaldehyde, then stained with 10 μ g/mL DAPI (2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride, Sigma), with PBS three times. Cells were observed and imaging was taken with a laser scanning confocal microscope (Leica TCS sp5, Germany).

In Vitro cell viability test

The effect of of MSNs-CADY and siRNA_{RANKL}/oestrogen/MSNs-CADY

on the viability of PDLSCs was tested by using the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium broide (MTT) assay. The PDLSCs were seeded in a 96-well plate at a density of 5000 cells per well. Thereafter, MSNs-CADY with different concentrations (200, 100, 50, 25, 12.5 and 6.25 mg/mL) were added to the medium, and then the cells were incubated for 24 and 48 h. Finally, the cell viability was assessed using the MTT assay.

Establishment of a model of Porphyromonas gingivalis infected PDLSCs

P.gingivalis was purchased from The Global Bioresource Center (ATCC, www.atcc.org), The strain was inoculated in fresh agar medium containing 5% sterile defibrized sheep blood, 1% hemin chloride and 0.1% vitamin K. Anaerobic culture at 37 °C for 5-7 days, monoclonal colonies were culated in liquid medium for 24 h, centrifuged for 10 min at 6000 g and 4 °C to collect bacteria. The bacteria were washed once by PBS and suspended in antibiotic-free cell culture medium. Then add 150 µL of 1×10^9 *P.gingivalis* into well cultured PDLSCs, all the test should be completed in 48 hours.

Characterization

The morphology and structure of nanoparticles were characterized by a field-emission scanning electron microscope (S-4800) at accelerating voltages of 5 and 200 kV.

Zeta potential measurements: Electrokinetic measurements were obtained at 25 °C between each functionalization step using a ZetaSizer Nano apparatus (Malvern Instruments, UK), in a PBS buffer solution (25 mM) at pH 7.2. The zeta potential was calculated using the Schmolukowski model.

Statistical Analysis

One-way ANOVA was used for statistical analysis of data to confirm the presence of significant differences in measured parameters between test groups; Individual points were compared using a Student's t test, and differences were considered significant at $p < 0.05$. All the experimental data were expressed as mean $\pm$ SD. All the experiments were repeated at least three times ($n = 3$).

Results

Preparation and Characterization of MSNs, MSNs-COOH, MSNs-CADY

The MSNs with the diameter about 120nm were first synthesized, then the black MSNs were modified with carboxyl, finally, cell penetrating peptide CADY was attached on the MSNs by use of EDC and NHS reaction. TEM images of each nanoparticle were shown in figure 1, and zeta potential of each nanoparticle indicated that the CADY has been modified on MSNs and 120 nm of MSNs-CADY was prepared successfully.

Cell Compatibility of MSNs-CADY

PDLSCs is relatively fragile, the cell compatibility of nano-drug carrier is very important. MTT cell activity detection kit was used to detect the effect of different concentrations of nanoparticles on cell viability, as shown in figure 2, MSNs-CADY has high PDLSCs compatibility, PDLSCs of cell viability was 94.5% and 88.4% even treated with 200 ug/mL MSNs-CADY for 24h and 48 h.

Cellular Uptake and Internalization of FITC-Labeled MSNs and FITC-Labeled MSNs-CADY

Suit nano-drug carriers should effectively carry drugs into cells, membrane-penetrating peptides such as CADY has been reported can improve the cellular internalization efficiency of drug-carriers [14-16].

Here, we constructed MSNs-CADY and then FITC was labeled on the surface of MSNs-CADY, 100 μ g/ml of FITC-labeled MSNs and FITC-labeled MSNs-CADY were separately incubated with PDLSCs for 12 h. As shown in figure 3, the FITC relative light density of FITC-Labeled MSNs was 13.4% and the FITC-Labeled MSNs-CADY 26.7%, the result indicated that CADY modification can significantly improve the efficiency of PDLSCs cellular uptake and internalization of nano drug carrier.

In Vitro Drug Releasing Property of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY

Mesoporous silica nanoparticles can be used as drug carriers because of their good sustained release properties, here, the in vitro drug releasing property of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY was tested, as shown in figure 4, MSNs-CADY has good releasing property to siRNA_{RANKL} and oestrogen both in siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY. After 10 h of rapid release, siRNA_{RANKL} and oestrogen loaded in MSNs-CADY can be released stably and slowly for at least 48 h. In addition, the MSNs-CADY prepared here has high drug loading capacity, 1mg of MSNs-CADY can load 24.6 mg oestrogen and 0.5 mM siRNA_{RANKL}.

Effect of siRNA_{RANKL}/oestrogen/MSNs-CADY on the cell proliferation of *P-gingivalis* infected PDLSCs

The direct effect of bacterial infection will cause to greatly reduction of cell proliferation. Here, we tested the effect of nano-drug carriers on the proliferation of *p-gingivalis* infected PDLSCs, as the result shown in figure 5, after 48h culture, the number of PDLSCs in control group, infected PDLSCs and infected PDLSCs+drug carrier group was about 445.7×10^4 , 117.9×10^4 and 390.5×10^4 separately, the results showed that siRNA_{RANKL}/oestrogen/MSNs-CADY can increase the proliferation of PDLSCs significantly.

Discussion

Periodontitis is a common complex disease with relatively late onset,

which is characterized by inflammatory destruction of periodontal attachment and bone loss. PDLSCs) are able to participate in connective tissue remodeling while suffering mechanical stress from orthodontic appliances. If the disease is not treated in time, repeated attacks over a long period of time can cause dental defects, and then affect oral function. In clinic, systemic or oral local therapy is often used [2, 17, 18]. The *P-gingivalis* is the main pathogen of periodontal disease [19]. It was reported that damage to periodontal ligament stem cells (PDLSCs) during illness is one of the reasons why the disease is difficult to eradicate [20, 21]. PDLSCs represent a unique mesenchymal stem cells (MSC) population as demonstrated by their capacity to generate a cementum/periodontal ligament - like tissue in vivo, highlighting their available and reliable features for periodontal regeneration. Previous research data showed that PDLSCs akin to the immunomodulatory properties exhibited by other stromal populations [4, 22], PDLSCs has been studied as the target of periodontitis treatment. Decreasing the expression of RANKL by using siRNA_{RANKL} in *P-gingivalis* infected PDLSCs is helpful for cells to maintain normal physiological functions [8]. Oestrogen (E2) also reported can enhanced the proliferation and osteogenic differentiation of hPDLSCs during in vitro culture. Both the siRNA_{RANKL} and oestrogen treatment of *P-gingivalis* infected PDLSCs need a drug-carrier which with good PDLSCs biocapacity, high drug

loading capacity and good drug release property, and the drug carrier also should have high cell internalization efficiency.

In this paper, MSNs-CADY was constructed to use as such nano-carrier, according to the results, MSNs-CADY prepared here having all the features required above, firstly, it has high cell compatibility, secondly, based on the mesoporous structure of mesoporous silica nanoparticles, the drug carrier has high siRNA_{RANKL} and oestrogen loading capacity, and good drug release property. After modification with cell penetrating peptide CADY, more MSNs-CADY can be taken by PDLSCs. In 2017, Lin et al reported that E2 was able to enhance the proliferation of human periodontal ligament stem cells [23], And in 2016, Jens Gruber et al delivered siRNA with polyoma JC virus-like particles can decrease the expression of RANKL in rats significantly [24]. Here, we studied the effect of siRNA_{RANKL} and oestrogen loaded MSNs-CADY on the cell proliferation of *P-gingivalis* infected PDLSCs, and results shown that siRNA_{RANKL}/oestrogen/MSNs-CADY can increase the proliferation of *P-gingivalis* infected PDLSCs significantly.

Conclusion

In short, siRNA_{RANKL}/oestrogen/MSNs-CADY we constructed here can significantly improve the cell proliferation of *P-gingivalis* infected PDLSCs, this nano drug carrier has the potential to be used in PDLSCs-based periodontitis treatment, this work provided a useful theoretical

basis and therapeutic ideas for the treatment of periodontitis. In addition, we will continue to evaluate the efficacy of the nano drug carrier in periodontitis treatment in vivo in the future and to provide preclinical theoretical basis for the treatment of periodontitis.

Abbreviations

PDLs: Periodontal ligament cells; PDLSCs: Periodontal ligament stem cells; MSC: Mesenchymal stem cells; MSNs: Mesoporous silica nanoparticles; *P.gingivalis*: *Porphyromonas gingivalis*; PDL: Periodontal ligament; RANKL/OPG: Receptor activator of NF- κ B ligand/osteoprotegerin; LPS: Lipopolysaccharide; E2: Oestrogen; TEOS: Tetraethoxysilane; APTES: 3-aminopropyl triethoxysilaneMethoxy; mPEG-SC: polyethylene glycol succinimidyl carbonate; DMSO: Dimethyl sulfoxide; DAPI: (2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; ANOVA: Analysis of variance.

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Availability of data and materials

All the data generated or analysed during this study are included in this published article.

Authors' contributions

Li Yan, and Liang Song was the head of this subject. Shi Xiaojun performed the design and management of experimental scheme and completed the preparation, testing and characterization of nanoparticles, Hu Fengling completed the culture of PDLCs. Chen Huijuan completed the imaging work by use of Laser confocal microscopy. Xu Bin completed the in vitro drug release of nanoparticle testing. Li Yinghua completed the MTT assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figure legends

Figure 1 Preparation and Characterization of MSNs, MSNs-COOH, MSNs-CADY.

Figure 2 Internalization characteristics of FITC-labeled MSNs and FITC-labeled MSNs@CADY. Blue fluorescence indicates nuclear staining with DAPI; green fluorescence indicates the localization of FITC-labeled MSNs, and red fluorescence indicates the staining of lysosomes by LysoTracker Red DND-99.

Figure 3 In Vitro Drug Releasing Property of siRNA_{RANKL}/MSNs-CADY and oestrogen/MSNs-CADY.

Figure 4 Cell Compatibility of MSNs-CADY.

Figure 5 The effect of MSNs/CADY/LRG on PDLSCs cell proliferation.

Figures

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Figures

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