

Attenuation of Inflammatory Response by 25-hydroxyvitamin D₃-loaded Polylactic Acid Microspheres in Treatment of Periodontitis in Diabetic Rats

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Objective: To investigate the inhibitory effects of 25-hydroxyvitamin D₃ (25(OH)D₃)-loaded polylactic acid (PLA) microspheres on inflammatory response in diabetic periodontitis.

Methods: 25(OH)D₃-loaded PLA microspheres were produced using emulsion-solvent evaporation method. Rat bone marrow stromal cells (BMSCs) cultured with *Aggregatibacter actinomycetemcomitans* in high glucose medium were chosen to mimic diabetic periodontitis. After cultivation with 25(OH)D₃-loaded PLA microspheres, inflammation-related proteins in BMSCs were detected using Western blot analysis. Periodontitis was induced using silk ligatures in diabetic rats, and 25(OH)D₃-loaded PLA microspheres were placed into the periodontal pockets. Periodontal tissues were examined using hematoxylin and eosin staining and western blot analysis.

Results: Drug release from the 25(OH)D₃-loaded microspheres was relatively steady during 70 days. In a diabetic periodontitis-like environment, 25(OH)D₃-loaded microspheres upregulated vitamin D receptor expression, and downregulated nuclear factor- κ B expression and signal transducer and activator of transcription 3 phosphorylation in the BMSCs. These 25(OH)D₃ microspheres also attenuated periodontal inflammatory infiltrate and bone loss in diabetic rats with periodontitis.

Conclusion: 25(OH)D₃-loaded microspheres could ameliorate diabetic periodontitis by inhibiting inflammatory response, and may provide a potential therapy for patients with this disease.

Key words: 25-hydroxyvitamin D₃, microsphere, inflammatory response, diabetes, periodontitis

Diabetes is a heterogeneous group of metabolic disorders that are characterised by hyperglycemia. Both major types of diabetes (type 1 and type 2) share similar complications. Periodontitis superimposed on diabetes, also known as diabetic periodontitis, prevails widely among diabetes sufferers¹. It often causes severe

damage of the alveolar bone, and has been considered as an important complication of diabetes².

An excessive inflammatory response in the periodontium is accepted as the key contributor to periodontal damage in diabetes with periodontitis³. Periodontal infection with hyperglycemia is believed to be the crucial causative factor for the exacerbated inflammatory response^{4,5}. Periodontal pathogens can produce toxins to stimulate the inflammatory response in periodontal tissues, including inflammation-related protein expression and inflammatory cell recruitment; high blood glucose affects these biological processes and aggravates the inflammatory state^{3,4}. Although the occurrence and development of periodontitis is associated with various pathogens, *Aggregatibacter actinomycetemcomitans* (*A.a.*) has been implicated as a causative agent of severe periodontitis in humans. It produces several factors

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contributing to the ability to colonise the oral cavity and cause disease, and has been used for mimic periodontitis *ex vivo*⁵.

Recently, 25-hydroxyvitamin D₃ (25(OH)D₃) has been found to be important in the regulation of inflammatory responses⁶. It is the stable form of vitamin D₃ in the body. Its deficiency is associated with amplified inflammatory states, including diabetes, and its supplementation promotes the attenuation of aggravated inflammatory reactions⁷. It can be hydroxylated by different periodontal cells and exert the biological functions through binding to the vitamin D receptor (VDR)⁶. After binding, VDR can regulate the expression or activation of inflammation-related proteins, and influence the development of inflammatory diseases^{8,9}. This research suggests the regulatory effect of 25(OH)D₃ on the inflammatory response in diabetic periodontitis and its potential in the treatment of this disease.

The biological effect of 25(OH)D₃, however, can not be sustained for long. It can be metabolically inactivated by various cells, and expression of the enzyme for 25(OH)D₃ degradation is always enhanced upon its addition, thus, its half-life is short in the body¹⁰. The modulation of inflammatory states in chronic diseases requires frequent and long-period vitamin D supplementation, and the regeneration of periodontal tissues often lasts over 10 weeks in diabetes¹¹⁻¹³. Thus, it may have great value to develop a sustained-release system to obtain a long-term effect of 25(OH)D₃ in periodontal tissues in the management of diabetic periodontitis. To date, there have been few reports on the sustained delivery system for 25(OH)D₃ application, but the technique for drug release over extended periods of time has been investigated for years. Microspheres of biodegradable polymers like polylactic acid (PLA) have been considered useful for therapeutic agent delivery in a sustained-release manner¹⁴. PLA microspheres can effectively reduce drug degradation before it exerts biological functions¹⁵. The total dose of medication and the characteristics of microspheres including particle size can be optimised¹⁶. Additionally, PLA does not exhibit untoward reactions either locally or systemically, when used in therapeutic applications^{16,17}. As such, the use of PLA microspheres may prolong the activity of 25(OH)D₃ and keep a long-term therapeutic effect in diabetic periodontitis.

In this study, 25(OH)D₃-loaded PLA microspheres were prepared and characterised. The effect of these microspheres on inflammatory response was examined in both *ex vivo* and *in vivo* diabetic periodontitis environments to test a therapeutic approach for treatment of this disease.

Materials and methods

Preparation of 25(OH)D₃-loaded microspheres

Microspheres were produced using the emulsion-solvent evaporation method¹⁰. A 4% (w/v) PLA (molecular weight of 200 kDa, Daigang Biomaterial) solution in dichloromethane (5 ml) was prepared. Amounts of 25(OH)D₃ (Sigma-Aldrich) were dissolved in the organic phase to make the drug/PLA ratio as 1:4. Afterwards, the organic phase was dropped into 100 ml aqueous solution of PVA (degree of hydrolysis 88%, Daigang Biomaterial), and stirred at 1,000 rpm. The prepared microspheres were collected by centrifugation and lyophilised. The same procedure without 25(OH)D₃ addition was used to obtain drug-free microspheres.

Characterisation of the produced microspheres

Lyophilised microspheres were assessed using a scanning electron microscope (SEM) (Jeol JSM-5400 LV, Jeol). Average diameter was calculated through examining 100 particles. Amounts of the produced microspheres were dissolved in 20 ml dichloromethane, and 25(OH)D₃ in this solution was analysed using UV/Vis spectrophotometer (U-3900H, Hitachi) at 265 nm. Drug content (% w/w) and encapsulation efficiency (%) were calculated from the following expressions¹¹:

- % Drug Content = (Drug in microspheres)/(Mass of microspheres) × 100 (1)
- % Encapsulation Efficiency = (Actual amount of drug in microspheres)/(Theoretical amount of drug in microspheres) × 1 (2)

To detect the release property, 10 mg 25(OH)D₃-loaded microspheres were suspended in 6 ml phosphate-buffered saline (PBS, pH 7.4) in tubes shaking at 60 rpm at 37°C. The tubes were centrifuged at predetermined time intervals, and 5 ml supernatant per tube was taken and replaced with 5 ml fresh buffer. The 25(OH)D₃ concentration of the withdrawn was detected spectrophotometrically at 265 nm. For morphological detection, 25(OH)D₃-loaded microspheres were subjected to the degradation procedure above, and the residue after centrifugation was scanned using SEM.

25(OH)D₃-loaded microsphere treatment in a diabetic periodontitis-like environment

Six male Wistar Hanover rats (100-120 g) were purchased from Dossy Co (Chengdu, China). The study on rat cells was approved by Ethics Committee of State Key

Laboratory of Oral Diseases, Sichuan University. The bone marrow stromal cells (BMSCs) were obtained as previously reported¹⁸. In brief, both sides of rat femora and tibiae were clipped off. Bone marrow was flushed out, filtered and centrifuged. The collected residue was resuspended with α -modified minimum essential medium (α -MEM, GibcoBRL, Grand Island, NY) containing 10 (v/v)% of penicillin/streptomycin and 10% of foetal bovine serum (GibcoBRL, Grand Island, NY).

A cultivation with *A.a.* was chosen to mimic periodontitis *ex vivo*¹⁹, and α -MEM with 12 mmol/L glucose was prepared to mimic the diabetic environment²⁰. *A.a.* strain ATCC 29522 was obtained from State Key Laboratory of Oral Diseases of Sichuan University (Chengdu, China). One week before *A.a.* addition, all BMSCs were incubated in the α -MEM without antibiotics in 6-well plates. Then the BMSCs were divided into four groups: normal control (N), diabetic periodontitis without treatment (DP), drug-free microsphere-treated diabetic periodontitis (FDP), and 25(OH)D₃-loaded microsphere-treated diabetic periodontitis (VDP) (approximately 3×10^6 BMSCs per group). An addition of *A.a.* in DP, FDP and VDP groups was followed¹⁹. Briefly, *A.a.* was added to cell cultures at 37°C at an m.o.i. (bacteria to BMSCs) of 10:1. In the presence of *A.a.*, BMSCs were incubated at 37°C in 5% CO₂ for 1 h, while normal control cells were inoculated with fresh sterile *A.a.* growth medium and maintained under the same conditions. After this incubation, BMSCs in all groups were washed with PBS and processed for the following experiments. N group was cultivated in α -MEM without antibiotics, and the other groups were cultivated in the high glucose medium (α -MEM with 12 mmol/L glucose). Afterwards, the media of FDP and VDP groups were supplemented with corresponding microspheres (2.5 \times 10⁻² g/L microspheres in the medium), and maintained for 3 days (all diabetic groups were maintained in the high glucose medium for 3 days). All experiments were carried out in triplicate.

Western blot analysis of inflammation-related proteins

After incubation with microspheres, the BMSCs of all groups were washed with PBS. Total protein was extracted and western blot analysis was carried out as described previously²¹. Primary antibodies were anti-GAPDH (1:300), anti-VDR (1:200), anti-nuclear factor- κ B (NF- κ B) (1:500), anti-signal transducer and activator of transcription 3 (STAT3) (1:200) and anti-phosphorylated STAT3 (pSTAT3) (1:200). Secondary antibodies were anti-rabbit (1:3000) or anti-mouse (1:3000) horse-

radishperoxidase-conjugated antibodies. All antibodies were from Santa Cruz Biotechnology.

Diabetic periodontitis rats with 25(OH)D₃-loaded microsphere treatment

All *in vivo* studies were approved by the Ethics Committee of State Key Laboratory of Oral Diseases, Sichuan University. Forty male Wistar Hanover rats (160–180 g) were obtained from Dossy (Chengdu, China). They were randomly divided into N, DP, FDP and VDP groups. There were 10 rats per group. N rats consumed low-fat standard chow (Dossy). DP, FDP and VDP rats consumed high-fat food (Dossy) for 2 weeks, and received an intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich) (35 mg/kg)²². One week after STZ treatment, the rat tail veins were pierced by a needle. Blood from the tails was obtained, and was used to determine the fasting blood glucose level by a glucometer (OneTouch Glucometer, LifeScan). The rats with the fasting blood glucose level above 13.89 mmol/L were considered diabetic²². Upon confirmation of diabetes, all rats were fed the standard chow, and silk ligatures were placed around both maxillary second molars of the diabetic rats²³. Ligatures were removed 4 weeks after placement, and the periodontal pocket depth of the maxillary second molars was examined using a periodontal probe. Then the drug-free and drug-loaded microspheres were placed into the periodontal pockets of FDP and VDP rats, respectively, at a dose of 1 mg/mm pocket depth.

Histological examination of periodontal tissues

All rats were sacrificed and the maxillae were harvested 70 days after microsphere application. Then the maxillae were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin²⁴. Sagittal paraffin sections were prepared at 5 μ m and examined using hematoxylin and eosin (H&E) staining. The number and the depth of polymorphonuclear leukocytes (PMNs) in gingiva and alveolar bone loss were determined in the sections as described previously²⁴. Five slides were used for each sample at 20 intervals, and both sides of maxillae were investigated. The averages of these measurements of both sides were calculated to represent the sample.

Statistical analysis

Differences in parameter mean values were analysed using one-way analysis of variance (ANOVA) testing followed by SNK-*q* multiple comparisons. Statistical significance was accepted at the $P < 0.05$ level.

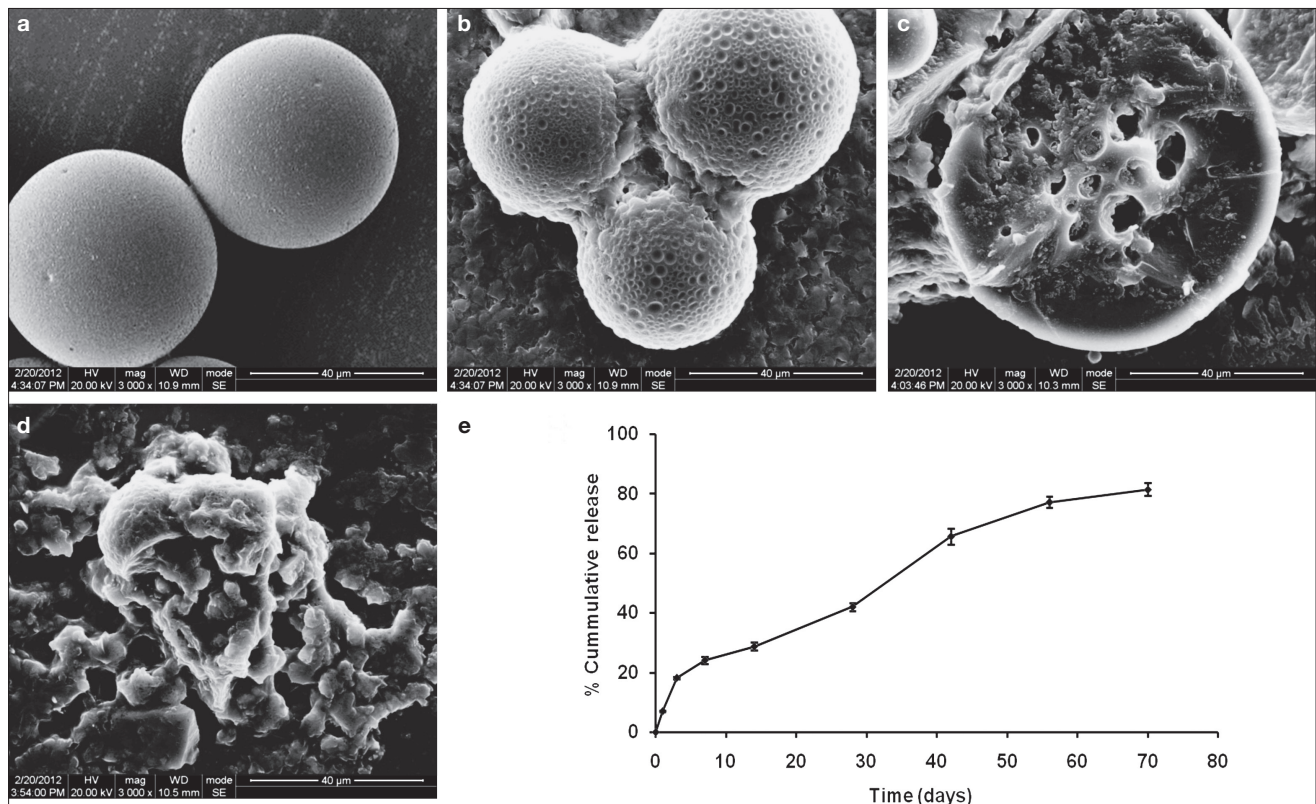


Fig 1 Morphology of 25(OH)D₃-loaded microspheres was examined using scanning electron microscopy. **a** 25(OH)D₃-loaded microspheres were spherical, and had intact surfaces before degradation. **b** After incubation in PBS (pH 7.4) at 37°C for 14 days, the microsphere surface became rougher and showed obvious pitting. **c** After incubation for 56 days, the microspheres had many pores on the surface, and lost their spherical shapes. **d** After incubation for 70 days, the microspheres collapsed leaving only eroded polymeric structures. **e** Drug release profile showed relatively steady 25(OH)D₃ release from the microspheres during 70 days.

Results

Characterisation of 25(OH)D₃-loaded microspheres

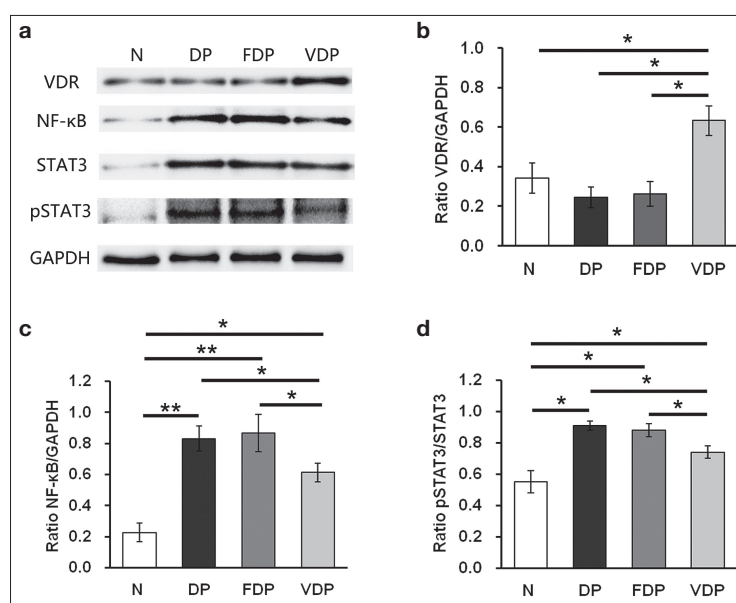
The prepared 25(OH)D₃-loaded microspheres were spherical in shape with intact surfaces (Fig 1a). The average diameter was (42.3 ± 5.8) μm; the drug content and encapsulation efficiency were (15.8 ± 0.5)% and (79.2 ± 1.9)%, respectively (values were shown as means ± SD, n = 3). Drug release from the PLA microspheres followed a near-to-zero-order release pattern, and was sustained over 70 days (Fig 1e). Degraded 25(OH)D₃-loaded microspheres exhibited increased surface roughness initially, followed by obvious surface pitting and pores, and finally collapsed, leaving only eroded polymeric structures (Figs 1b to 1d).

Effect of the prepared microspheres on inflammation-related protein expressions

To evaluate the effect of 25(OH)D₃-loaded microspheres on inflammatory response *ex vivo*, the expres-

sion of VDR, NF-κB, STAT3 and pSTAT3 in BMSCs was detected using western blot analysis. VDR protein level in 25(OH)D₃-loaded microsphere-treated group was obviously elevated compared with that in the other three groups. There were no significant differences among the N, DP and FDP groups (Fig 2). As shown in Fig 2, the DP, FDP and VDP groups had dramatically enhanced NF-κB levels compared to the N group, and the DP and FDP groups had higher levels of NF-κB expression compared to the VDP group. No significant difference was found between the DP and FDP groups. The ratio of pSTAT3/total STAT3 increased in the DP, FDP and VDP groups compared with that in the N group, suggesting greater activation of STAT3 under the condition of diabetic periodontitis. Upon 25(OH)D₃-loaded microsphere treatment, this ratio significantly decreased (VDP vs DP, and VDP vs FDP), while it exhibited no significant change after drug-free microsphere treatment (FDP vs DP), suggesting the reduction of STAT3 phosphorylation by 25(OH)D₃-loaded microspheres.

Fig 2 Protein expressions of VDR, NF- κ B, STAT3 and pSTAT3 in BMSCs were examined using western blot analysis. The treatment with 25(OH) D_3 -loaded microspheres enhanced VDR protein levels, reduced NF- κ B expression, and inhibited STAT3 phosphorylation. N: normal control; DP: diabetic periodontitis without treatment; FDP: drug-free microsphere-treated diabetic periodontitis; and VDP: 25(OH) D_3 -loaded microsphere-treated diabetic periodontitis. The data were expressed as means \pm SD (n = 3). * $P < 0.05$ and ** $P < 0.01$.



Effect of the microspheres on periodontal inflammatory infiltrate and bone loss

The number and the depth of PMNs in gingival connective tissue have been accepted to demonstrate inflammatory infiltrate in periodontal diseases. It was seen in Fig 3 that the number and the depth of PMNs were greater in the gingival connective tissue in the DP, FDP and VDP groups, compared to the N group. They did not differ between the DP and FDP groups, while they were obviously reduced upon 25(OH) D_3 -loaded microsphere treatment (VDP vs DP, and VDP vs FDP).

Periodontal bone resorption has been linked to the inflammatory cell infiltrating to deeper areas of periodontal tissues, and the amount of alveolar bone loss is also widely used to represent the severity of periodontal inflammation. In this experiment, alveolar bone loss was seen in all groups with diabetic periodontitis, compared to the normal group (DP vs N, FDP vs N, and VDP vs N), suggesting the periodontal inflammation in the diabetic periodontitis model. Furthermore, the amount of bone loss was dramatically decreased after 25(OH) D_3 -loaded microsphere treatment (VDP vs DP, and VDP vs FDP), but there was no significant difference between DP and FDP groups (Fig 3), suggesting the inhibition of inflammatory response in the periodontium by 25(OH) D_3 -loaded microspheres.

Discussion

In this study, 25(OH) D_3 -loaded PLA microspheres were prepared using emulsion-solvent evaporation method.

The produced microspheres exhibited relatively high encapsulation efficiency, and almost steady drug release during the 70-day degradation. The release profile may be explained by the degradation property of PLA microparticles. Previous researches have shown that the degradation of PLA microparticles includes water penetration into the microsphere matrix and the following bulk hydrolysis of ester bonds²⁵. As the polymer chains inside the matrix are cleaved by hydrolysis, the microsphere erosion and the matrix porosity increases, and therefore the drug release by diffusion occurs²⁶. With the long-period degradation of PLA microspheres, the slow drug release can be observed. The present release profile showed a fast release of 25(OH) D_3 in the initial stage, which may be associated with a relatively large amount of drug located close to the microsphere surface. The drug near the surface can be released by diffusion before significant polymer degradation happens²⁶.

To examine the effect of the 25(OH) D_3 -loaded microspheres on inflammatory response in diabetic periodontitis *ex vivo*, *A.a.*-infected BMSCs under the condition of high glucose were used. Periodontal cells cultured with high glucose and periodontal pathogens have been chosen to mimic diabetic periodontitis *ex vivo*. BMSCs are one of the important periodontal tissue cells. They can express inflammation-related proteins, and recruit immune cells, and are widely used in researches on pro-inflammatory state of diabetes and periodontal infection^{27,28}. As the main circulating form of vitamin D_3 metabolites in the body, 25(OH) D_3 can be hydroxylated into 1,25-dihydroxyvitamin D_3 by 1α -hydroxylase, which exists in various cells^{6,9}. 25(OH) D_3 acts on VDR

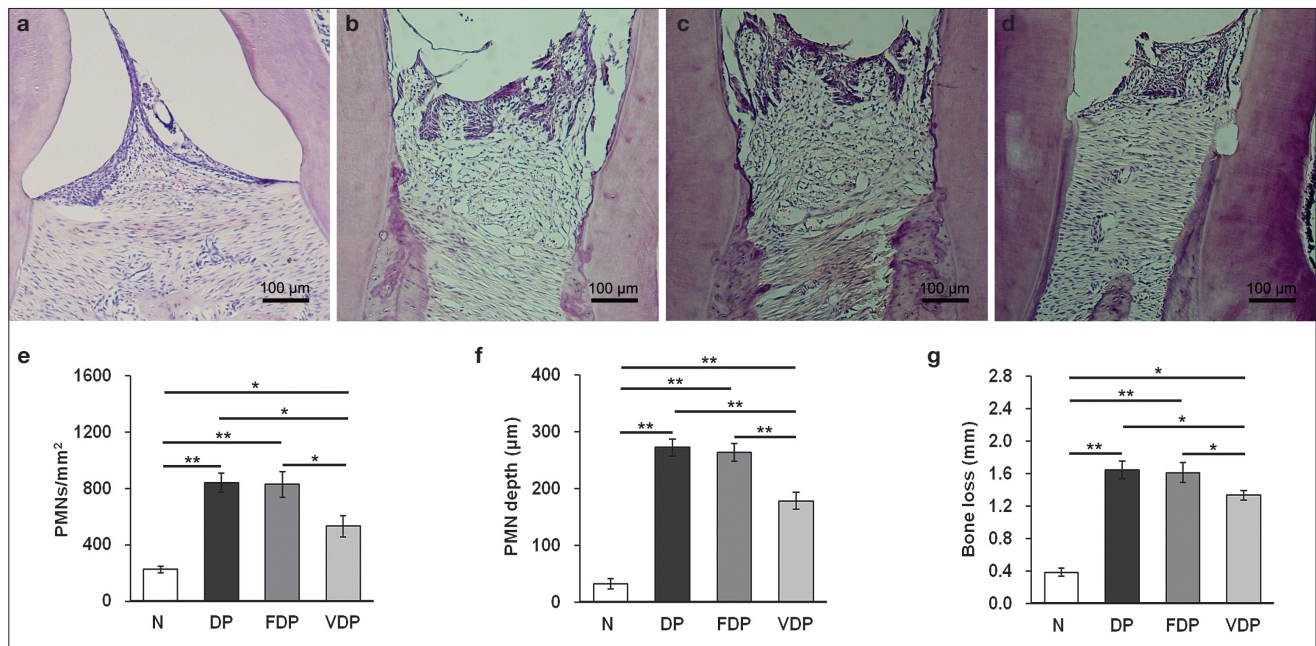


Fig 3 Inhibition of the inflammatory response in the periodontium by 25(OH)D₃-loaded microspheres. The mid-interproximal region between first and second maxillary molars was examined in each rat specimen in H&E-stained sections (a-d). The 25(OH)D₃-loaded microsphere treatment reduced the number of PMNs and the depth of PMNs in the gingiva of diabetic rats with periodontitis (b-f). Alveolar bone loss in the diabetic rats with periodontitis was decreased upon 25(OH)D₃-loaded microsphere treatment (g). N: normal control; DP: diabetic periodontitis without treatment; FDP: drug-free microsphere-treated diabetic periodontitis; and VDP: 25(OH)D₃-loaded microsphere-treated diabetic periodontitis. The data were expressed as means ± SD (n = 10). * p<0.05 and ** p<0.01.

directly or after hydroxylation, causing the change of downstream pathways like NF-κB and STATs signaling to exert biological functions, including attenuation of inflammatory response^{8,9}.

Western blot analysis in this experiment showed that the 25(OH)D₃ microsphere-treated BMSCs exhibited enhanced VDR expression, suggesting that the 25(OH)D₃ released from microspheres could generate biological responses in these cells. It has been demonstrated that different cells including kidney and bone cells can express 1α-hydroxylase and hydroxylate 25(OH)D₃²¹. The hydroxylated 25(OH)D₃ (1,25-dihydroxyvitamin D₃) promotes the expression of VDR, and binds to this receptor, and subsequently regulates the downstream signaling²⁹. There are several other important proteins in VDR-mediated signaling, including CYP27B1 and CYP24A1. These two proteins always exist in cells which express VDR. As the key vitamin D₃ metabolising enzyme, CYP27B1 converts 25(OH)D₃ to its active form 1,25(OH)₂D₃. 25(OH)D₃ treatment can increase both CYP27B1 and VDR expression, and regulate other proteins in the VDR signaling, and enhance the function of vitamin D330. Expression of CYP24A1, a major vitamin D₃ catabolising enzyme, can be also increased after 25(OH)D₃ addition in differ-

ent cells^{30,31}. It degrades 25(OH)D₃ and 1,25(OH)₂D₃, inhibits their long effect, and serves as a biomarker for VDR activation³². Whether the expression of these two enzymes changed upon the microsphere application was unclear at this point. Further experiments are needed to test the effects.

Moreover, an increase in NF-κB expression was seen in all diabetic periodontitis groups compared with the normal group, while the expression was suppressed upon 25(OH)D₃ microsphere treatment. NF-κB is a key transcription factor greatly involved in the excessive inflammatory response³³. The abnormal activation of NF-κB signaling can be observed in a variety of inflammatory diseases including diabetic periodontitis, and the NF-κB activation is enhanced in periodontal tissues in this disease³⁴. It has been reported that VDR can affect the function of NF-κB DNA binding motif, and suppress NF-κB protein synthesis in various cells^{8,9}. Through impacting VDR, vitamin D₃ metabolites can reduce NF-κB expression and subsequently inhibit NF-κB signaling, an important VDR downstream pathways, and finally attenuate inflammatory response⁸.

Additionally, enhanced phosphorylation of STAT3 was observed in the BMSCs cultured in the diabetic periodontitis-like environment, while it decreased

upon 25(OH)D₃-loaded microsphere application. These results suggested the inhibition of STAT3 activation by the 25(OH)D₃ release from microspheres. Current reports highlight the strong association between STAT3 phosphorylation and inflammatory response^{35,36}. Activated NF-κB/STAT3 signaling in BMSCs promotes immune cell infiltrate, a key process in inflammatory response and tissue damage in the periodontium; suppression of NF-κB/STAT3 activation can be observed after the amelioration of periodontitis and diabetic complications^{37,38}. Vitamin D₃ metabolites may suppress the NF-κB expression and STAT3 phosphorylation and downstream signalings, and inhibit the excessive inflammatory reaction and periodontal destruction. In this work, no significant change of the expression of inflammation-related proteins was found between the drug-free microsphere-treated group and the untreated diabetic periodontitis group, suggesting that PLA microspheres, as a drug carrier, do not regulate the inflammatory response.

To further assess the inhibitory effect of 25(OH)D₃-loaded microspheres on inflammatory response, diabetic rats with periodontitis were used in this study. The high-fat diet combined with STZ injection in rats is accepted to induce diabetes models, and tying ligatures around molars is considered as a commonly used method for establishing periodontitis animal models^{23,39}. Ligature placement can effectively facilitate bacterial invasion of periodontal tissues and cause periodontitis in diabetic rats^{3,23}.

H&E staining showed that the PMN infiltrate in gingiva was greater in all diabetic periodontitis rats compared with the normal controls, whereas the infiltrate was reduced upon 70-day 25(OH)D₃-loaded microsphere treatment. These data suggested the attenuation of the inflammatory response by the long-period release of 25(OH)D₃ from microspheres. The vitamin D₃ metabolites have a short half-life in the body, so the supplementation generally lasts several months in the treatment of chronic diseases^{11,12}. PLA microspheres are widely used carriers for drugs including steroids and proteins¹⁵. As the PLA polymer in microspheres degrades, drug-release and therapeutic effects can be sustained over the long term. Previous studies have found that the infection with hyperglycemia enhances the expression of NF-κB and pSTAT3, and therefore promotes PMN recruitment in the local tissue^{27,40}. As the crucial immune cells in the development of diabetic periodontitis, infiltrating PMNs can further promote inflammatory response including inflammatory cytokine overexpression and activation, leading to periodontal damage like connective tissue destruction

and bone resorption⁴¹. The number of PMNs and the depth of PMNs in the gingiva in periodontitis are representative of the severity of periodontal inflammatory response. The 25(OH)D₃ supplementation can affect the inflammatory proteins like NF-κB and pSTAT3, and subsequently alter the downstream signaling, resulting in the amelioration of PMN infiltrate³⁶.

Meanwhile, histological examination showed a decrease in alveolar bone loss caused by diabetic periodontitis upon 25(OH)D₃ microsphere treatment, and no significant difference between the drug-free microsphere-treated group and the untreated diabetic periodontitis group. These observations also suggested that the sustained-release of 25(OH)D₃ from microspheres could suppress the inflammatory response in the periodontium. It has been demonstrated that the infiltrating PMNs in the periodontium can produce pro-inflammatory proteins including tumor necrosis factor α and interleukin⁶, contributing to alveolar bone loss in the diabetic periodontitis⁴². Thus, the alveolar bone loss is also representative of the severity of inflammatory response in the periodontal tissue. Inflammation-related pathways involved in PMN infiltrate including NF-κB/STAT3 signaling can be inhibited by vitamin D₃ metabolites, so excessive inflammatory reactions and alveolar bone loss can be reduced³⁶. This study demonstrated the long-term efficacy of 25(OH)D₃-loaded microspheres and the great promise in the treatment of diabetic periodontitis. But further studies are needed to elucidate the effect of these microspheres on the inflammatory response and other biological functions *in vivo*.

In this work, 25(OH)D₃-loaded microspheres were successfully prepared and exhibited long-term 25(OH)D₃ release. These microspheres could modulate the expression of inflammation-related proteins in the *ex vivo* diabetic periodontitis environment, and inhibit the inflammatory infiltrate and alveolar bone loss in the diabetic periodontitis rats. These results suggest that the 25(OH)D₃-loaded microspheres could attenuate the inflammatory response in diabetic periodontitis and provide an effective approach for the treatment of this disease.

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