25-Hydroxyvitamin D₃ Alleviates Experimental Periodontitis via Promoting Expression of Cathelicidin in Mice with Type 2 Diabetic Mellitus

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Summary Type 2 diabetic mellitus is manifested by metabolic impairments with high prevalence worldwide, of which periodontitis represents a typical oral complication (also called diabetic periodontitis). Oral epithelia bear the brunt of periodontal damage from microscopic intruders; thus the defense function of epithelial cells is of vital significance. We have previously proved that 25-hydroxyvitamin D₃ (25-OHD₃) altered the expression of cathelicidin antimicrobial peptide in oral epithelial cells in vitro. Herein, we discovered that 25-OHD₃ intraperitoneal injection attenuated periodontal inflammation by promoting cathelicidin production in gingival epithelia and reducing fasting glucose of diabetic mice. Dotblotting of serum showed cathelicidin secretion was consistent with 25-OHD₃ treatment. Immunohistochemistry exhibited enhanced expression of cathelicidin and vitamin D receptors along with reduced expression of TLR4 in diabetic mice. Stereomicroscope showed less alveolar bone loss when injected with 25-OHD₃. These results showed 25-OHD₃ can promote cathelicidin and ameliorate the severity of diabetic periodontitis. Our study complemented the mechanism of cathelicidin and extended knowledge of 25-OHD₃'s role in diabetic periodontitis.

Key Words cathelicidin, 25-hydroxyvitamin D₃, type 2 diabetic mellitus, periodontitis, oral epithelia

Type 2 diabetic mellitus represents a metabolic disorder of high prevalence all over the world. It is characterized by impaired fasting glucose and glycosylated hemoglobin (1). Periodontitis is one typical complication, also known as diabetic periodontitis (2). Patients suffer from severe periodontal inflammation and an accelerated alveolar bone loss rate while initial periodontal therapy cannot provide prognosis equivalent to that for those without diabetes (3). Vitamin D₃ was previously elaborated as a negative regulator in diabetic periodontitis with potent experimental promise.

Gingival epithelia comprise of the first line of defense against periodontal pathogenic bacteria. However, the oral cavity provides a complicated condition for its constant exposure to a variety of microorganisms; thus the integrated cell line is particularly significant. Either weakened protective ability or excessive defense response can contribute to the breakdown of the innate immunity (4, 5). Periodontitis takes place when cell membrane receptors on gingival epithelial cells detect pathogens (6). Once activated, Toll-like receptor 4 (TLR4) transmits signals to cytoplasmic adaptors and activates proinflammatory pathways like the NF- κ B pathway (7). Inflamed periodontal tissues are

prone to express high levels of TLR4 and cytokines like tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1) (8, 9). Vitamin D₃ has been found to both directly influence TLR4 expression and indirectly modulate cell response to TLR4 ligands (10–12), and finally improves outcomes of patients with chronic diseases. The biological effects of vitamin D₃ are carried out by transforming to its main circulating form, 25-hydroxyvitamin D₃ (25-OHD₃), which undergoes another hydroxylation to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and combines with vitamin D receptor (VDR), both of which are verified to take place in gingival epithelia (13, 14). We have proved that two vital enzymes, CYP27B1 and CYP24A1, take charge of transforming 25-OHD₃ to its active form, 1,25(OH)₂D₃, and the degradation of 1,25(OH)₂D₃, respectively, in gingival epithelia. Meanwhile, the balance between two enzymes can violently influence the function of vitamin D₃ (14).

Vitamin D₃ initiates its antibiotic function through the stimulation of antimicrobial peptides, including cathelicidin antimicrobial peptide (CAMP) (15, 16). Cathelicidin is primarily produced by phagocytes and epithelial cells, and exerts biological functions by mediating defense against microbial invasion. It can kill microorganisms directly and induce chemotaxis and promote chemokine release (17, 18). Aside from its role in augmenting inflammation, it has been discovered to

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promote wound healing as an adjuvant (19). Besides, high levels of cathelicidin expression were observed after knocking out the inactive enzyme of vitamin D₃, which was explicitly elaborated in our previous work (14). Although the underlying mechanism has not been largely understood, cathelicidin as an effective immune adjuvant should be considered in seeking a novel therapy for diabetic periodontitis. Thus the potential activator, 25-OHD₃, with its astonishing outcomes, may be beneficial and economical for patients. We hypothesized that 25-OHD₃ supplementation could promote cathelicidin production and ameliorate periodontitis in mice with type 2 diabetes.

MATERIALS AND METHODS

Mice and in vivo model. Thirty 5-wk-old male BKS. Cg-Dock7m^{+/+}Leprdb/Nju mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The mice were kept in a constant-temperature room with 12 h light-dark cycles for SPF animals, and fed with standard laboratory food (Dossy Co., Chengdu, China) and sterile water. They were randomly divided into 3 groups: diabetes group (D group), diabetic periodontitis group (DP group, orally inoculated) and diabetic periodontitis group treated with 25-OHD₃ (DPV group). All animals were sacrificed at 18 wk old. The whole study was conducted strictly according to the Animal Ethics Procedures and Guidelines of the People's Republic of China and approved by the Animal Ethics Committee of Sichuan University (Permit No. WCCSIRB-D-2015-075).

Experimental periodontitis induction. *Porphyromonas gingivalis* (P.g.) strain W50 was kindly provided by the State Key Laboratory of Oral Diseases of Sichuan University (Chengdu, China) and grown anaerobically in blood-agar (Oxoid, Oxoid Ltd., Hampshire, England) with hemin/menadione (Sigma-Aldrich, Sigma-Aldrich Co., St. Louis, MO). At 8 wk old, the mice in the DP and DPV groups were orally inoculated with P.g. three times every other day within 5 d as follows: 10⁹ colony-forming units of live bacteria were dispersed in 100 μ L phosphate buffered saline (PBS) containing 2% carboxymethylcellulose. Mice in the D group were given the same volume of PBS with 2% carboxymethylcellulose.

Intraperitoneal treatment with 25-OHD₃. Mice in the DPV group were intraperitoneally injected with 25-OHD₃ (Sigma-Aldrich Co.) at a dose of 5 μ g/kg body weight every 2 d from 9 wk-age till the day before sacrifice. 25-OHD₃ was dissolved in refined peanut oil (Sigma-Aldrich Co.), which was made freshly. Mice in the control groups (D and DP group) received the same volume of refined peanut oil intraperitoneally.

Fasting blood glucose and body weight analysis. Food was removed in advance to guarantee 10 h fasting blood glucose. Mouse tail veins were pierced to determine the fasting blood glucose levels with a glucometer (OneTouch Glucometer, LifeScan, Milpitas, CA). Weight was measured with a weighing scale accurate to 0.01 g. Measurements were carried out at 6 wk of age for a baseline record, and every week after each treatment

and before sacrifice.

Enzyme-linked immunosorbent assay (ELISA). At sacrifice, blood for ELISA analysis was collected after fasting glucose levels were detected as above. The blood was centrifuged to remove cells, and serum was collected for vitamin D₃ level and IL-1 determination with corresponding ELISA kits (Zcibio, Shanghai, China, and Invitrogen, Waltham, MA, respectively). The cross reactivity in 25-OHD₃ ELISA kit is less than 8.85% with 24,25-(OH)₂D₃ and 9.76% with 1,25-(OH)₂D₃. Experiments were conducted according to the protocol in triplicate.

Dot blot analysis. The serum was collected from mice tails at 6, 7, and 8 wk of age to record cathelicidin levels coupled with each treatment. The terminal serum was collected as mentioned before. For dot blot analysis, 5 mm*5 mm grids were drawn on a 0.45 μ m nitrocellular membrane (Bio-Rad, China) and 4 μ L of each sample was slowly loaded onto the center of the grid. Then the membrane was blocked by soaking 5% BSA in TBS-T for 1 h at room temperature. Anti-CAP18 (1 : 500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA) was used for incubation overnight in 4°C. After the membrane was washed with TBS-T three times, secondary antibody (Boster, China) was used to incubate with the membrane for 1 h. Detection was carried out using Chemiluminescent Substrate System (Biorad, China).

Quantification of alveolar bone loss. Mouse mandibular jaws were dissected from surrounding soft tissues after sacrifice. Alveolar bone loss was monitored by calculating the first and second molars using a stereomicroscope with attached digital camera (Leica MZ FLIII, Leica, Wetzlar, Germany). The area bordered by the cemento-enamel junction, the alveolar bone crest, and the mesial and distal line angles on the lingual side of the mandibular first and second molars was measured to define the bone loss level. The measurements were blindly conducted on 15-fold magnified pictures in triplicate.

Immunohistochemical analysis. Mouse maxillas were collected at sacrifice. Then they were prepared and examined using immunohistochemical staining. In brief, the maxillas were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) (BioRad, BioRad Laboratories, Hercules, CA) solution for 30 d, and then embedded in paraffin. Five micrometers sliced sections were made and detected using immunohistochemical staining. Five slides were used for each sample at 10 intervals. Both sides of maxillas were studied and controls were made with PBS incubation instead of the primary antibodies. The primary antibodies, anti-VDR (1 : 50), anti-TLR4 (1 : 200), and anti-CAP18 (1 : 100), and the secondary antibodies (1 : 1,000) were all from Santa Cruz Biotechnology. Dab staining was carried out using a dab staining kit (KeyGen, China) following the manufacturer's instructions. The staining images were taken using a microscope with an attached digital camera (Nikon 80i, Nikon Ltd., Tokyo, Japan).

Statistical analysis. Data were calculated and given as mean $\pm$ SE. Differences about fasting blood glucose and weight and bone loss area were analyzed using one-

Fig. 1. Fasting blood glucose levels and weight of D, DPV and DP mice were examined corresponding to each treatment during disease. (A) All treatments were applied to *db/db* mice strictly according to the procedure. Measurements and serum sample collection were carried out at 6 wk age, 8 wk age, 9 wk age and 18 wk age. (B) Fasting glucose level showed a similar growing pattern before 25-OHD₃ was taken out, whereas DPV mice had lowest glucose level compared to other groups. (C) DP mice and D mice exhibited continuous growth in weight while DP mice had least mean weight at sacrifice. The difference of weight between DP and DPV mice was not statistically significant. Values are means $\pm$ SE ($n=10$). ** $p<0.01$ for D vs. DP mice and * $p<0.05$ for D vs. DPV mice and DP vs. DPV mice.

way analysis of variance (ANOVA) followed by SNK- q multiple comparisons. Student's t -test was used for testing the role of vitamin D₃ supplementation on cathelicidin production on the basis of a difference between two means (25-OHD₃ vs. peanut oil control) and whether any of those means were statistically significantly different from each other. The Chi square test was used for examining the differences between positive expression rates among all groups from immunohistochemistry data. Statistical analyses were performed using SPSS 22.0 for Windows. Statistical significance was defined as $p<0.05$.

RESULTS

Glucose level and body weight varied in accordance with treatments

Fasting blood glucose and weight were recorded in order to investigate the effect of 25-OHD₃ on glucose status and mouse growth during disease (Fig. 1A). Fasting blood glucose and weight were observed to be similar at 6 wk of age and recorded as a baseline. After 1 wk for quarantine, mice in the DPV group and DP group were infected *P.g.* orally while the D group received a control treatment. *P.g.* infection impaired weight but not fasting

glucose level in the DPV group and DP group according to the results. Fasting blood glucose increased largely after the animals' adaption and remained consistent among all groups (Fig. 1B). The impact of experimental periodontitis on mice health accounted for the reduction in weight. The followed week after the infection procedure ceased, 25-OHD₃ injection was carried out every other day. DPV mice showed lowest glucose level and a small weight recovery (Fig. 1C), which may be owing to both the halt of oral infection and the influence of 25-OHD₃. However, DP mice had the highest glucose level and the lowest average weight while the D group showed a steady increase in fasting glucose and weight. The measurements were taken for the last time before termination. 25-OHD₃ was observed exerting influence on both fasting blood glucose and weight. The D group and DP group had a similar high glucose level compared to the DPV group (Fig. 1C, $p=0.044$ and $p=0.025$ respectively). Meanwhile, weight in the D group and DPV group rose sharply compared with the DP group ($p=0.008$ and $p=0.013$ respectively).

25-OHD₃ supplementation ameliorated alveolar bone loss

Each maxillary alveolar bone was observed under the

Fig. 2. Alveolar bone loss was calculated based on the lingual side of the mandibular first and second molars of D, DP and DPV mice. (A–C) Black lines are drawn around the area bordered by the cemento-enamel junction, the mesial and distal line angles and the alveolar bone crest of the mandibular first and second molars. Area within the line was measured as alveolar bone loss in D, DP and DPV groups accordingly. (D) The bone loss of *db* mice with experimental periodontitis was the greatest among all groups. A significant decrease in bone loss area was observed in DPV mice compared to DP mice. Values are means $\pm$ SE ($n=10$). ** $p<0.01$ for D vs. DP mice, D vs. DPV mice and DP vs. DPV mice.

Fig. 3. Serum vitamin D₃ levels and IL-1 levels between DP and DPV groups were measured using ELISA at the time of sacrifice. (A) Vitamin D₃ levels were increased in DP mice that had accepted 25-OHD₃ treatment. (B) IL-1 levels were increased in DP mice compared to DPV mice. Values are means $\pm$ SE ($n=10$). ** $p<0.01$ for DPV vs. DP mice.

stereomicroscope. Horizontal observation showed that the alveolar bone of all groups had absorption of different degrees. Alveolar bone loss in the D group (Fig. 2A) and DPV group (Fig. 2C) revealed little root surface on average. DP mice lost much more alveolar bone whose absorption extended to root furcation and resulted in teeth displacement (Fig. 2B). After quantitative analysis of the bone resorption area, we found that the alveolar bone loss was the most advanced in the DP group compared with the D group and DPV group (Fig. 2D, $p<0.001$). Mice with 25OHD₃ supplementation were

observed to have experienced ameliorated bone resorption compared to the DP group ($p=0.005$).

VD₃ and IL-1 level in the DPV group showed observable changes

ELISA kits were used to detect the vitamin D₃ level and IL-1 level of all mice after sacrifice. Elisa detection of vitamin D₃ is considered to be highly effective and accurate, but vitamin D₃ involves all its metabolic products, including 1,25(OH)₂D₃ and 25-OHD₃. Nevertheless, 25-OHD₃ represents the most stable product and its circulating form and comprises most of vitamin D₃. In

Fig. 4. Cathelicidin levels in serum and in periodontal tissues. (A) Dot blotting results of all groups examined from serum collected at each detecting time point. Grey values were calculated to analysis the difference between DP mice and D mice to evaluate the influence of vitamin D₃. Circulating cathelicidin in two groups exhibited continually growing trend during disease. After 25-OHD₃ injection treatment finished, cathelicidin in DPV mice was greater than that in DP mice. (B) Cathelicidin expression in the mouse alveolar bone ridge crest and the gingival epithelium was shown in the immunohistochemical images above. Elevated expression of cathelicidin was observed in DPV and DP groups, while DPV group showed enhanced expression. * $p < 0.05$ for DPV vs. DP mice and ** $p < 0.01$ for DPV vs. DP mice.

this study, total vitamin D₃ was measured to make sure of a better calculation of both stable stored vitamin D₃ and that activated to exert biological effects. The vitamin D₃ level in terminal blood serum was estimated and the results were as follows. Diabetic periodontitis mice with vitamin injection had higher vitamin D₃ and there was a significant difference (Fig. 3A, $p = 0.001$). On the

other hand, 25-OHD₃ supplementation was accompanied with a sharp reduction of IL-1 level in diabetic mice with periodontitis. However, the IL-1 level in the DP group was obviously higher than in the other groups (Fig. 3B, $p = 0.005$).

Fig. 5. Protein expression of cathelicidin, VDR and TLR4 in the mouse alveolar bone ridge crest and the gingival epithelium was shown in the immunohistochemical images. Elevated expression of VDR was observed in DPV group, while DP group showed decreased expression compared to D group. Mice that had accepted periodontal infection exhibited higher expression of TLR4. DPV group had decreased TLR4 expression compared to that of DP mice.

Cathelicidin production in serum and in oral epithelia was elevated after 25-OHD₃ treatment

Dotblotting was carried out to examine 25-OHD₃'s role in cathelicidin production in general, using serum samples from the DPV and DP groups which were centrifuged as declared before. Cathelicidin, a secretory protein, was observed to vary in accordance with *P.g.* infection and 25-OHD₃ injection. Baseline serum showed no observable differences between the two groups, but *P.g.* infection had increased cathelicidin production in all groups with the DPV group in the lead ($p=0.013$). It was possible that individual differences led to this significant difference, but cathelicidin in the DP group was observed to have increased in the following week. 25-OHD₃ clearly promoted cathelicidin to a large extent (Fig. 4A). Although terminal serum showed a similar outcome, the differences were still of statistical significance ($p=0.006$). Additionally, an immunohistochemistry test on local gingival epithelia had the same result. As has been shown, cathelicidin was expressed among all groups but there was a clear difference (Fig. 4B). Cathelicidin was observed mostly in cytoplasm, and dab staining of cathelicidin from the DPV group showed the most positive keratinized cells. Gingival epithelia from the DP group exhibited a higher proportion than from the D group.

25-OHD₃ supplementation increased VDR expression and decreased TLR4 expression in periodontal tissues

VDR and TLR4 expression was detected by immunohistochemistry. The results coincided with our previous experiment. VDR is important for 25-OHD₃ function and following VDR-related element transcription and it has been verified to be expressed in various cells, including oral keratinocytes. We observed that VDR was expressed mostly in the nucleus of gingival epithelial cells but also in cytoplasm, and that TLR4 were mostly expressed on the membrane surface (Fig. 5). VDR expression was up-regulated when supplemented with additional 25-OHD₃. The DP group had the lowest VDR expression and the highest TLR4 expression compared to others. Although TLR4 protein detected in the DPV group showed a higher level than in the D group, there was still a clear decrease compared with the DP group.

DISCUSSION

In this study, the diabetic mice with periodontal infection showed a severely inflamed gingival condition and weaker health with enhanced TLR4 expression and IL-1 level, suggesting augmented recognition and response to periodontal pathogens as a synergistic effect of diabetes. Interestingly, cathelicidin secretion, for defending against danger and invasion and consolidating the innate immune system (20), was found to vary according to inflammation severity. It could be assumed that

inflammation itself was a valid boost for cathelicidin production. After an experimental periodontitis model was successfully exhibited, general secretion of cathelicidin was elevated as determined from collected serum samples. Vitamin D₃ has been verified as an anti-inflammatory regulator and its supplementation showed corresponding results. 25-OHD₃ injection not only alleviated inflammation by reducing TLR4 expression and IL-1 production (11, 12, 21), but also increased cathelicidin secretion.

Periodontitis with diabetes often involves severe manifestations and poor outcomes, which increases both the health burden and clinical difficulties (22, 23). The underlying mechanism of aggravated inflammation may be attributed to a defective innate immune system under hyperglycemia. As the first defense line, oral epithelia provide physical and biological shields against harmful intrusions; thus the integration of epidermal covering is essential to maintain anti-infection capability (24). Enhanced TLR4 signaling could be a vital mechanism behind the tissue damage associated with diabetic periodontitis. If activated, its intracellular domain adapts to the adaptor and leads to further activation of downstream proinflammatory proteins (25). Diabetic mellitus and its representative vessel inflammation exacerbate microcirculation disturbances (26); as a consequence, periodontal tissue becomes easily infected which is even more serious than for those without diabetes (27). In this case, an alternative and economical method that can both enhance resistance to infection and alleviate periodontal destruction is of crucial significance.

An impaired immune system requires a boost for immunoenhancement. The oral cavity is constantly exposed to various pathogenic microorganisms, demanding adequate and moderate immune protective molecules (28, 29). Antimicrobial peptides, also known as cationic host defense peptides, are expressed to defend against pathogenic microbes by the host (30). Cathelicidin, a homolog of human LL-37 in mice, has been recognized as a potential therapeutic treatment for infection (31). LL-37 was elevated in patients with T2DM and general periodontitis and was positively correlated with inflammatory markers (32). Considering the fact that diabetic mice with *P.g.* infection all showed elevated cathelicidin production, it could be presumed that cathelicidin and its protective function in patients with chronic inflammation weren't adequate for defending against infection. Despite its excellent characteristics like anti-inflammation and wound healing promotion, its potential toxicity and risks in disturbing the flora balance indeed restrict the commercial application (14, 33); thus a novel strategy is required to boost its production properly.

Expressions of several antimicrobial peptides including cathelicidin are reported to be influenced by vitamin D₃ (34, 35). The underlying mechanism came with the function of VDR and VDR response elements in the promoter of the CAMP gene. CYP27B1 takes charge of converting 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃, which leads to the binding of the

1,25(OH)₂D₃-VDR-RXR heterodimer to the VDR elements of the cathelicidin gene (34). Aside from directly exerting its antimicrobial effect, cathelicidin is reported to inhibit *Escherichia coli*-induced TLR4 activation in a viability-dependent manner (35). This indicates that cathelicidin functions only when bacterial viability is lost, thus giving a reasonable explanation for its inadequacy in chronic diseases. Our study ascertained that 25-OHD₃ promoted the production of cathelicidin in serum and in gingival tissues in a moderate manner, along with the downregulation of TLR4. It could be inferred the effect of 25-OHD₃ was achieved by promoting cathelicidin, thereafter influencing TLR4 and its pro-inflammatory function.

Our previous study found that OKF/TERT2 cells could elevate CAMP levels by inhibiting the expression of CYP24A1, which is required to initiate the side chain degradation of vitamin D₃ metabolites, while the break of the balance between CYP24A1 and CYP27B1 can cause resistance to 25-OHD₃ therapy (14). Terminated CYP24A1 function led to blocking the transformation of 25-OHD₃ to 24,25-(OH)₂D₃ and 1,25-(OH)₂D₃ to 1,24,25-(OH)₃D₃. Having verified altered cathelicidin levels followed by CYP24A1 knock out in vitro, in this study we aimed to explore whether 25-OHD₃ application accompanied mild inflammation and increased cathelicidin levels in vivo. We chose to lay more attention on the changes in periodontitis with diabetes after boosting 25-OHD₃ intake because of the previous background. Therefore, we chose to measure the alteration of serum 25-OHD₃ instead of other vitamin D₃ metabolites. Expression of cathelicidin is dependent on sufficient vitamin D₃ levels. Patients with chronic intestinal disease who accept additional vitamin D₃ treatment experienced higher levels of cathelicidin and alleviated mucosal destruction (36). The 25-OHD₃ dosage is based on our previous work, which successfully established experimental animal models of diabetic periodontitis without toxic reaction. Moderate vitamin D₃ intake should be regarded as a necessary and economical way to promote cathelicidin secretion and to ameliorate periodontal inflammation under hyperglycemia.

In this study, we have to admit there are still some inadequacies. Despite all procedures being carefully and correctly implemented, periodontal tissues from diabetic mice with periodontitis were severely hampered; thus sections became extremely vulnerable during antigen retrieval. Cathelicidin secretion was in consistence with 25-OHD₃ supplementation; its production in terminal serum from mice without treatment was nevertheless elevated. It has been put forward that general chronic inflammation can induce cathelicidin secretion (32, 37), which may reflect the need of the organism to fight against pathogens and danger. We suggested that mice in the experiment all shared a common hyperglycemia baseline with periodontitis, which already influenced general cathelicidin levels, leading to elevated cathelicidin that concealed 25-OHD₃ effects. 25-OHD₃ has its own pharmacokinetic and mechanistic features, but it may vary slightly in *db* mice, therefore requiring further

experiments to ascertain an appropriate injection concentration. On the other hand, we shall work on discovering a proper time for blood collecting to avoid missing the peak 25-OHD₃ value.

In conclusion, this study demonstrates the expression of elevated cathelicidin in the gingival epithelium of diabetic mice with periodontitis. Additional vitamin D₃ intake notably alleviated periodontal tissue destruction. The observation suggests an alternative mechanism by which application of 25-OHD₃ eases the inflammation in the periodontal tissue and elevates cathelicidin to stabilize immune function in the oral cavity. Thus, daily vitamin D intake should be recommended as an effective and economical method to modulate immune function by enhancing cathelicidin production.

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