Expression of vitamin D 1α-hydroxylase in human gingival fibroblasts *in vivo*

Kaining Liu [1, 2, 3, 4], **Bing Han** [2, 3, 4, 5], **Jianxia Hou** [1, 2, 3, 4], **Jianyun Zhang** [2, 3, 4, 6], **Jing Su** [7], **Huanxin Meng** [Corresp. 1, 2, 3, 4]

[1] Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China

[2] National Clinical Research Center for Oral Diseases, Beijing, China

[3] National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing, China

[4] Beijing Key Laboratory of Digital Stomatology, Beijing, China

[5] Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing, China

[6] Department of Oral Pathology, Peking University School and Hospital of Stomatology, Beijing, China

[7] Department of Pathology, Peking University Health Science Center, Beijing, China

Corresponding Author: Huanxin Meng
Email address: kqhxmeng@bjmu.edu.cn

Background. Vitamin D 1α-hydroxylase CYP27B1 is the key factor in the vitamin D pathway. Previously, we analyzed the expression of CYP27B1 in human gingival fibroblasts *in vitro*. In the present study, we analyzed the gingival expression of CYP27B1 *in vivo*.
Methods. Forty-two patients with periodontitis Stage IV Grade C and 33 controls were recruited. All patients with periodontitis had unsalvageable teeth and part of the wall of the periodontal pocket was resected and obtained after tooth extraction. All controls needed crown-lengthening surgery, and samples of gingiva resected during surgery were also harvested. All the individuals' gingivae were used for immunohistochemistry and immunofluorescence. In addition, gingivae from seventeen subjects of the diseased group and twelve subjects of the control group were analyzed by real-time PCR.
Results. Expression of CYP27B1 was detected both in gingival epithelia and in gingival connective tissues, and the expression in connective tissues colocalized with vimentin, indicating that CYP27B1 protein is expressed in gingival fibroblasts. The expression of CYP27B1 mRNA in gingival connective tissues and the CYP27B1 staining scores in gingival fibroblasts in the diseased group were significantly higher than those in the control group.
Conclusions. Expression of CYP27B1 in human gingival tissues was detected, not only in the fibroblasts of gingival connective tissues, but also in the gingival epithelial cells, and might be positively correlated with periodontal inflammation.

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[5] Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing, China

[6] Department of Oral Pathology, Peking University School and Hospital of Stomatology, Beijing, China

[7] Department of Pathology, Peking University Health Science Center, Beijing, China

*** Corresponding Author:**

Huanxin Meng

Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, PR China. E-mail address: corresponding_author kqhxmeng@bjmu.edu.cn

Abstract

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Results. Expression of CYP27B1 was detected both in gingival epithelia and in gingival connective tissues, and the expression in connective tissues colocalized with vimentin, indicating that CYP27B1 protein is expressed in gingival fibroblasts. The expression of CYP27B1 mRNA in gingival connective tissues and the CYP27B1 staining scores in gingival fibroblasts in the diseased group were significantly higher than those in the control group.

Conclusions. Expression of CYP27B1 in human gingival tissues was detected not only in the fibroblasts of gingival connective tissues, but also in the gingival epithelial cells, and might be positively correlated with periodontal inflammation.

Introduction

Vitamin D_3 is of great importance in regulating calcium and phosphorus metabolism and immunological responses (Sassi et al., 2018; Medrano et al., 2018). The active hormonal metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25OH_2D_3$), is formed by two-step hydroxylations (Medrano et al., 2018): ① from vitamin D_3 to 25-hydroxyvitamin D_3 ($25OHD_3$) by vitamin D 25-hydroxylase in the liver, followed by ② from $25OHD_3$ to $1,25OH_2D_3$ by vitamin D 1α-hydroxylase in the kidney. Vitamin D 1α-hydroxylase CYP27B1 was first detected in the kidney (Takeyama et al., 1997), but subsequently extra-renal sites of $1,25OH_2D_3$ synthesis were also verified, including the skin (Bikle & Christakos, 2020), prostate (Capiod et al., 2018), bone (van Driel et al., 2006), eye (Alsalem et al., 2014; Markiewicz et al., 2019), blood vessels (Somjen et al., 2005; Zehnder et al., 2002), human periodontal ligament cells and human gingival fibroblasts (hGFs) (Liu et al., 2012a; Liu et al., 2012b).

The vitamin D pathway, including connected reactions from the activation of toll-like receptors to the expression of the human cationic antimicrobial protein of 18 kDa (hCAP18) in monocytes, was first proposed in 2006 (Liu et al., 2006). hCAP18 is the precursor of the important antimicrobial peptide, cathelicidin (composed of 37 amino acids, also called LL37), which is the end product of the vitamin D pathway (Liu et al., 2006). LL37 has a broad-spectrum antibacterial effect, and has a regulatory effect on the immuno-inflammatory response (Teles et al., 2013; Xhindoli et al., 2016; Wang et al., 2019). A similar pathway also exists in keratinocytes (Schauber et al., 2007). In our previous study (Gao et al., 2018), the vitamin D pathway was detected in hGFs, and vitamin D 1α-hydroxylase CYP27B1 was demonstrated to be

the key factor in the pathway. Our results suggested that the vitamin D pathway might be important in periodontal immune defense (Gao et al., 2018), which was in line with another research group (Zhou et al., 2018). As the key factor in the vitamin D pathway (Gao et al., 2018), CYP27B1 is worthy of further research. To our knowledge, however, the *in vivo* expression of CYP27B1 in hGFs has not been reported.

Although CYP27B1 expressed in hGFs *in vitro* is the same as that in kidney, its regulation is different: periodontitis-related inflammatory stimuli interleukin-1β (IL-1β), sodium butyrate and *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS) induce significant up-regulation of CYP27B1, while regulators of 1α-hydroxylase in kidney (parathyroid hormone, calcium and $1,25OH_2D_3$) do not significantly influence the expression of CYP27B1 in hGFs *in vitro* (Gao et al., 2018; Liu et al., 2012b). However, the actual situation *in vivo* is much more complicated than that simulated *in vitro*. Previously, our group reported that IL-1β and butyric acid, which are both up-regulators of CYP27B1, could be detected in the gingival crevicular fluids of patients with periodontitis, and the concentrations were positively correlated with periodontal inflammation (Liu et al., 2010; Lu et al., 2014). Thus, based on our previous data, it might be hypothesized that CYP27B1 is expressed in hGFs *in vivo* and patients with periodontitis might have stronger expression. The aim of this study was to test the above hypothesis and to elucidate the features of CYP27B1 expression in hGFs *in vivo*. In addition, the expression of CYP27B1 in human gingival epithelial cells (hGEs) was analyzed.

Materials & Methods

Tissue sampling

The institutional review board of Peking University School and Hospital of Stomatology approved the study protocol (PKUSSIRB-2011007). Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki.

Forty-two patients with periodontitis Stage IV Grade C and 33 healthy controls were enrolled from the clinic of the Periodontology Department, Peking University School and Hospital of

Stomatology. On the basis of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Tonetti et al., 2018; Lang & Bartold, 2018; Papapanou et al., 2018), diagnosis was made for each individual after complete periodontal examination. The inclusion criteria were as follows. Periodontitis: at least eight teeth with probing depth (PD) ≥ 7 mm and evidence of alveolar bone loss on radiographs; at least four teeth with mobility II or III; at least one unsalvageable tooth with mobility III and alveolar bone resorption close to the root apex, needing to be extracted. Healthy controls: no site with attachment loss (AL); no site with PD > 3 mm after supragingival scaling; no radiographic evidence of alveolar bone loss; less than 10% of sites with bleeding on probing (BOP); at least one tooth needing crown-lengthening surgery. Any subjects with systemic diseases or pregnancy were excluded. All 75 subjects enrolled were non-smokers.

The PD of all the enrolled subjects' teeth and AL of each unsalvageable tooth or each control tooth needing crown-lengthening surgery were recorded at six sites (mesial, distal, and middle sites of facial and lingual sides). Bleeding index (BI) (Mazza et al., 1981) was also recorded for each tooth of each individual. The mean PD, AL and BI were calculated for each analyzed tooth. The percentage of surfaces (facial and lingual) with BOP was also calculated and recorded as BOP%.

All unsalvageable teeth were extracted before periodontal treatment. After extraction of the unsalvageable teeth, part of the wall of the periodontal pocket was resected and harvested. The gingiva resected during the crown-lengthening surgery of the controls was also collected. Gingival samples from 17 subjects of the diseased group and twelve subjects of the control group were divided into part 1 and part 2. Gingival connective and epithelial tissues were obtained from part 1 using sharp tissue scissors, and then were stored in RNAwait (Solarbio Science & Technology Co., Beijing, China) at -80°C until RNA extraction. Part 2 and gingival samples from the other subjects were dehydrated and embedded in paraffin and serial sections were cut with the microtome set at 5 μm. One section of each sample was examined after staining with hematoxylin and eosin (H&E).

Detection of CYP27B1 expression

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, US) and was reverse transcribed to cDNA using a reverse transcription kit (TOYOBO Life Science (Shanghai), Shanghai, China). Real-time PCR reactions were performed using Faststart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) in a real-time Thermocycler (Applied Biosystems, Foster City, CA, US) in triplicate. β-actin was used as an internal control (Forward primer: 5'-GCCGTGGTGGTGAAGCTGT-3' and reverse primer: 5'-ACCCACACTGTGCCCATCTA-3'). The forward primer for detection of CYP27B1 was 5'-ACGGTGTCCAACACGCTCT-3' and the reverse primer was 5'-AACAGTGGCTGAGGGGTAGG-3'. Data are presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ (ΔCt = Ct of target gene minus Ct of β-actin) (Livak & Schmittgen, 2001).

Immunohistochemistry

Immunohistochemistry was performed according to previously described methods (Li et al., 2017). Briefly, selected sections were transferred onto adhesive slides (Zhongshan Golden Bridge Biotechnology Co., Beijing, China), deparaffinized with xylene and rehydrated with descending concentrations of ethanol, then digested with 1 g/L trypsin at 37°C for 10 min for antigen retrieval. Endogenous peroxidase blocking was achieved by treatment with 3% H_2O_2 for 10 min at room temperature, then sections were incubated with primary sheep polyclonal antibody against CYP27B1 (1:100; The Binding Site Ltd., Birmingham, UK) at 4°C for 12 h. This was followed by incubation with an anti-sheep secondary antibody (1:500; EarthOx Life Sciences, San Francisco, CA, US) at 37°C for 30 min. The PV-9000 Polymer Detection System and a diaminobenzidine (DAB) kit (both from Zhongshan Golden Bridge Biotechnology Co., Beijing, China) were used for immunohistochemical staining of CYP27B1. The DAB staining time was 150 s for each section. Finally, sections were counterstained with hematoxylin.

Immunofluorescence

After deparaffinization of the sections, antigen retrieval was accomplished by boiling in citric acid–sodium citrate buffer (0.01 M, pH 6.0) for 15 min, and endogenous peroxidase blocking was performed using the same method as described above. Then, sections were incubated with a

working solution of primary rabbit anti-vimentin monoclonal antibody (ZA-0511; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at 4°C for 12 h. Next, sections were incubated with an anti-rabbit secondary antibody (ZF-0511, diluted 1:400; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at 37°C for 1 h. Then sections were incubated with primary mouse monoclonal antibody against CYP27B1 (sc-515903, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, US) at 4°C for another 12 h. The anti-mouse secondary antibody (sc-516141, diluted 1:50; Santa Cruz Biotechnology, CA, US) was added and incubated at 37°C for 1 h, then nuclei were counterstained with DAPI (Neobioscience Biological Technology Co., Shenzhen, China), and sections were observed using immunofluorescence microscopy (Nikon, Tokyo, Japan).

Image analysis

Image evaluation of the immunohistochemical results was performed by two experienced pathologists, who were unaware to which group the histological sections belonged. The CYP27B1 staining of each sample was rated as one of the following four grades: negative (-), weak (+), moderate (++) or strong (+++), translated as 0, 1, 2 and 3 for statistical analysis, respectively. The staining intensity of each sample was the consensus of the opinions of the two pathologists.

Each pathologist chose five non-overlapping 40× microscope fields of each section for evaluation of hGFs. The total number of hGFs and the number of immunohistochemically CYP27B1-positive ones in each chosen microscope field were recorded and staining of gingival fibroblasts were rated as negative (-), weak (+), moderate (++) or strong (+++). The percentage of +, ++ or +++ hGFs was calculated and CYP27B1 staining score was calculated using the following formula: CYP27B1 staining score = (percentage of +++ cells) × 3 + (percentage of ++ cells) × 2 + (percentage of + cells). The method for calculating CYP27B1 staining score was previously reported by Yoon *et al.* (Yoon et al., 2010) and a higher score indicated stronger staining intensity. Cell counting and rating were performed by the two pathologists using a multi-person sharing microscope (Olympus, Tokyo, Japan) at the same time. They calculated the

185 average CYP27B1 staining score of each section, and the mean of the two average CYP27B1
186 staining scores for each section was used for analysis.
187 Since immunofluorescence was usually used for qualitative analysis, the results of
188 immunofluorescence staining were only used for the colocalization of vimentin and CYP27B1 in
189 hGFs.
190 **Statistical methods**
191 The Mann-Whitney U Test was used to compare AL, BI, BOP%, the relative mRNA expression
192 of CYP27B1 in gingival epithelia and the staining grades of the two groups since normal
193 distribution was not assumed. All the other comparisons between the two groups were carried
194 out using Independent-samples T Test. Statistical analyses were carried out using the SPSS 11.5
195 software package (SPSS Inc., Chicago, IL, US). A P value < 0.05 was considered statistically
196 significant.
197 All the parameters were used to calculate power values using PASS 2008 (NCSS Inc.,
198 Kaysville, UT, US) and each power value was over 0.99.
199
200 **Results**
201 The characteristics of the two groups are shown in Table 1. Significantly higher PD and AL were
202 observed in the periodontitis group than in the control group. BI and BOP% of all teeth analyzed
203 in the periodontitis group were 4 and 100%, respectively. BI of the teeth analyzed in the control
204 group was 0 or 1, so none had BOP.
205 The mRNA expressions of CYP27B1 in gingival connective tissues of patients with
206 periodontitis were significantly higher than those in the gingival connective tissues of controls
207 (Fig. 1A). In contrast, there was no significant difference in CYP27B1 mRNA expression
208 between the gingival epithelia of the two groups (Fig. 1B).
209 The gingiva of one patient with periodontitis (Fig. 2A–C) and one control (Fig. 2D–F) are
210 shown. Negative controls are shown in Fig. 2 (G–I). The black frame indicates the epithelial
211 tissue, and the blue frame indicates the connective tissue. As shown in the figure, the gingival

connective tissues were CYP27B1 positive, and the expression of CYP27B1 was also detected in gingival epithelia. In the periodontitis group, the expression of CYP27B1 was detected in all epithelial layers, but expression was stronger in the superficial layer than in the deep layer of the epithelia in the control group. As shown in Fig. 3 (A–F), the expressions of CYP27B1 and vimentin were colocalized, indicating that in gingival connective tissues, the cells positive for CYP27B1 expression were hGFs. Statistical analysis indicated that CYP27B1 staining intensities of the gingiva of patients with periodontitis [3.00, (3.00 to 3.00)] were significantly higher than those of the controls [1.00, (1.00 to 2.00)] (Fig. 4A). In all the 40× microscopic fields chosen for analysis, almost 100% of the hGFs were CYP27B1 positive and the CYP27B1 staining scores of hGFs of patients with periodontitis (2.49 ± 0.08) were significantly higher than those of controls (1.84 ± 0.12) (Fig. 4B).

Discussion

Our previous experiments *in vitro* verified that CYP27B1 is expressed in hGFs and that expression is up-regulated by the inflammatory stimuli, IL-1β and sodium butyrate (Liu et al., 2012b). In the present study, we demonstrated the expression of CYP27B1 in gingival connective tissues *in vivo*. Since there are several types of cells in gingival connective tissues, immunofluorescence experiments were performed and demonstrated that CYP27B1 colocalized with vimentin, a marker of fibroblasts, indicating that the CYP27B1-positive cells in gingival connective tissues were hGFs. However, it should be pointed out that endothelial cells also express vimentin (Piera-Velazquez & Jimenez, 2019), as shown in Fig. 3. Additionally, as shown in Fig. 2 and Fig. 3, endothelial cells were also found to be CYP27B1 positive, which was in line with the results of Zehnder *et al.* (Zehnder et al., 2002). Because hGFs and endothelial cells can easily be distinguished by pathologists, our morphological analysis of hGFs was not influenced by endothelial cells. Although the actual situation *in vivo* is much more complex than that simulated in the laboratory, our observations that patients with periodontitis had higher mRNA expression of CYP27B1 and higher CYP27B1 staining scores were in line with our results *in*

vitro. Therefore, our hypothesis that "CYP27B1 is expressed by hGFs *in vivo* and the expression might be positively associated with periodontal inflammation" was verified.

Although we focused on hGFs, epithelial tissues were also observed in the present study, and gingival epithelia were found to be CYP27B1 positive in both groups, which was in line with the findings of other researchers (McMahon et al., 2011; Menzel et al., 2019). What should be pointed out is that the distribution of CYP27B1 expression differed between the two groups (Fig. 3). When obtaining the gingival epithelial tissues for analysis of mRNA expression, it was impossible to obtain the entire epithelium clinically. Therefore, only the superficial layer was obtained to avoid contamination of connective tissues. Since CYP27B1 expression was relatively strong in the superficial layers in both groups, the lack of a significant difference in CYP27B1 expression between the epithelia of the two groups could be explained.

It has been demonstrated that a vitamin D pathway exists in hGFs (Gao et al., 2018) and hGEs (McMahon et al., 2011; Menzel et al., 2019). The pathway might be involved in periodontal immune defense for the following reasons. (1) $25OHD_3$ alleviates experimental periodontitis in diabetic mice via the vitamin D pathway (Zhou et al., 2018). (2) In hGFs, the pathway is activated by the periodontal inflammatory stimulus *Pg*-LPS, and suppresses the expression of some inflammatory chemokines such as IL-8 and MCP-1 (Gao et al., 2018), indicating that the pathway might play a role in immune defense in periodontal soft tissues. (3) $25OHD_3$ is an important part of the vitamin D pathway, and higher $25OHD_3$ concentrations were detected in both the gingival crevicular fluids and the plasma of aggressive periodontitis patients compared to those of healthy controls (Liu et al., 2009). Moreover, after periodontal inflammation is reduced by initial periodontal therapy, the $25OHD_3$ levels in gingival crevicular fluids and plasma significantly drop (Liu et al., 2010), indicating that activity of the vitamin D pathway might be positively associated with periodontal inflammation. As the key factor in the pathway (Gao et al., 2018), CYP27B1 is worthy of further research. In the present study, the finding that the *in vivo* gingival CYP27B1 expression was higher in the periodontitis group than in the control group could provide new evidence of the involvement of the vitamin D pathway in

periodontal immune defense. According to the study by Tada et al. (Tada et al., 2016), $1,25OH_2D_3$ stimulation resulted in over 70-fold up-regulation of hCAP-18/LL-37 in an hGE cell line (Ca9-22). In contrast, $25OHD_3$ or $1,25OH_2D_3$ stimulation only resulted in 3- to 4-fold enhancement of expression of hCAP-18/LL-37 in hGFs (Gao et al., 2018). As the forefront of periodontal immune defense, it is reasonable that hGEs had a more active vitamin D pathway than hGFs. However, the relatively less active vitamin D pathway in hGFs is still worth studying, because once hGE, as the first line of periodontal defense, is breached and periodontal inflammation exacerbates to include gingival connective tissues, hGFs could play their role in periodontal immune defense through the vitamin D pathway. Thus, the present study is of biological significance, although more mechanisms via which the vitamin D pathway impacts gingival health in periodontitis still need to be elucidated.

Reasons for the higher expression of CYP27B1 in the periodontitis group might be as follows: (1) $25OHD_3$ is an up-regulator of CYP27B1 in hGFs (Gao et al., 2018), and $25OHD_3$ levels in gingival crevicular fluids of patients with periodontitis before initial periodontal therapy were significantly higher than those after therapy (Liu et al., 2010); (2) Periodontal inflammation results in higher concentrations of IL-1β and butyric acid in gingival crevicular fluids (Liu et al., 2010; Lu et al., 2014), which also induces the expression of CYP27B1 in hGFs (Liu et al., 2012b).

Our previous studies (Liu et al., 2009; Liu et al., 2010) indicated that systemic and local $25OHD_3$ levels in patients with aggressive periodontitis were positively associated with periodontal inflammation. However, several existing studies (Dietrich et al., 2004; Jimenez et al., 2014; Zhan et al., 2014) suggested that vitamin D deficiency is associated with an increased risk of periodontal disease. What should be pointed out is that in these studies, the participants were about 50 years of age or older, an age range that did not overlap with that of the population in our previous studies (younger than 30 years old). Additionally, no correlation between plasma $25OHD_3$ levels and periodontal health was found in another large cross-sectional study (Antonoglou et al., 2015), and the participants in that study were 30-49 years old. Thus, the

relationship between $25OHD_3$ and periodontitis in people of different ages might be different.

In studies investigating the association between $25OHD_3$ and periodontal health in large samples (Dietrich et al., 2004; Jimenez et al., 2014; Zhan et al., 2014; Antonoglou et al., 2015), the participants were from the general population. However, in our previous study (Liu et al., 2009), only patients with aggressive periodontitis had higher plasma $25OHD_3$ levels, and the patients had much more severe periodontal inflammation than the other participants. In the special group, it is unclear whether the higher plasma $25OHD_3$ level is the reason for or the result of severe periodontal inflammation. Our previous study (Gao et al., 2018) suggested that $25OHD_3$ activates the vitamin D pathway, which participates in periodontal immune defense. Therefore, it is possible that, due to severe periodontal inflammation, more LL37 is needed for antibacterial and anti-inflammatory function, and more $25OHD_3$ is synthesized for the more active vitamin D pathway in periodontium. This possibility could help to explain why patients with severe periodontitis had higher systemic and local 25OHD3 levels. In the present study, the results that patients with periodontitis had higher CYP27B1 expression in hGFs indicated that patients with periodontitis had a more active vitamin D pathway in hGFs, which further supported this possibility.In the present study, subjects matched by age and gender were included in the two groups and all were non-smokers, in order to minimize the influence of potential confounding factors. To analyze the typical inflammatory situation *in vivo*, all the patients enrolled were diagnosed with periodontitis Stage IV Grade C, the most severe periodontitis in the new classification scheme for periodontal diseases (Tonetti et al., 2018; Papapanou et al., 2018). In addition, all gingival tissues of patients with periodontitis were obtained around unsalvageable teeth, which had not received any periodontal therapy so that periodontal inflammation was serious enough and was not influenced by periodontal treatments. The PD and AL of the unsalvageable teeth analyzed were high and BOP was positive at all surfaces of the teeth. In contrast, all the teeth analyzed in the control group had PD less than 3 mm and had no AL or BOP, indicating that these teeth were clinically healthy. It should be pointed out that all the teeth analyzed in the control group needed crown-lengthening surgery because of excessive

gingival display or subgingival location of fracture lines or carious lesions. When parts of the teeth were subgingival, accumulation of dental plaque was often detected. Thus, the BI of some teeth in the control group was 1 and mild inflammation of the gingiva could be detected. Similarly, it was reported that "healthy" gingiva might also harbor inflammatory cellular infiltrates, indicating that subclinical gingivitis might exist (Lang & Bartold, 2018). Thus, CYP27B1 staining intensities of two of the 33 teeth in the control group were strong (+++) and the mild inflammation of the gingiva might be the reason for high expression of CYP27B1 in the control group.

Immunohistochemistry is of course a highly subjective method. We tried to objectively evaluate CYP27B1 expression in gingiva *in vivo* by letting two experienced pathologists perform the evaluation in a blinded manner. However, the subjectivity of the evaluation was inevitable, which is a limitation of the present study.

Conclusions

In summary, CYP27B1 expression was detected in hGFs *in vivo*, and this expression might be induced by periodontal inflammation. These results validated our previous *in vitro* findings, and indicated the potential involvement of the vitamin D pathway in periodontal immune defense. The present study can help lay the foundation for using vitamin D pathway in the treatment of periodontitis via vitamin D supplement.

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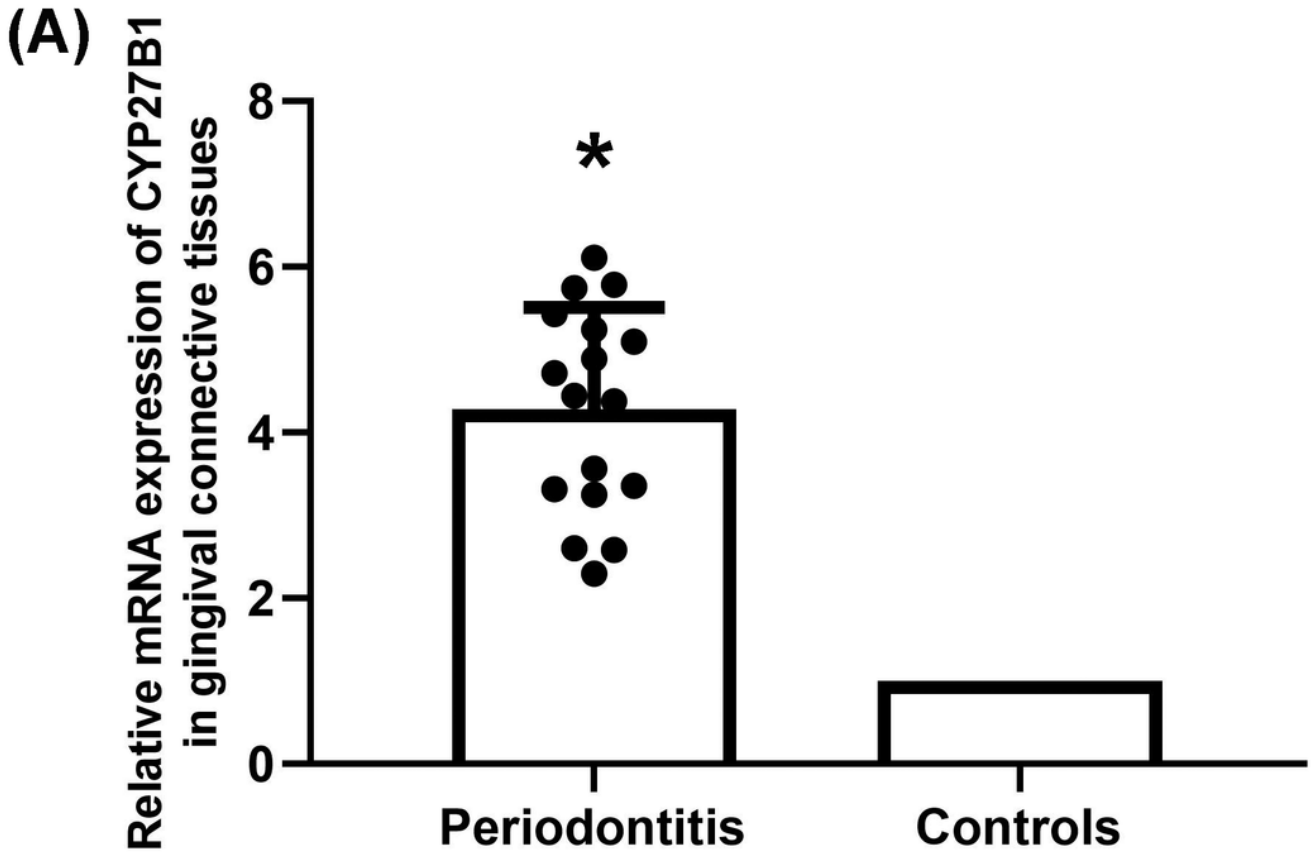
Figure 1

Fig. 1 Expression of CYP27B1 mRNA in gingiva

(A) Expression of CYP27B1 mRNA in gingival connective tissues of the diseased group was significantly higher than in the control group. (B) Expression of CYP27B1 mRNA in gingival epithelia did not significantly differ between the two groups.

* Compared to the control group, $P < 0.05$.


(A)
Relative mRNA expression of CYP27B1 in gingival connective tissues
8
6
4
2
0
*
Periodontitis
Controls

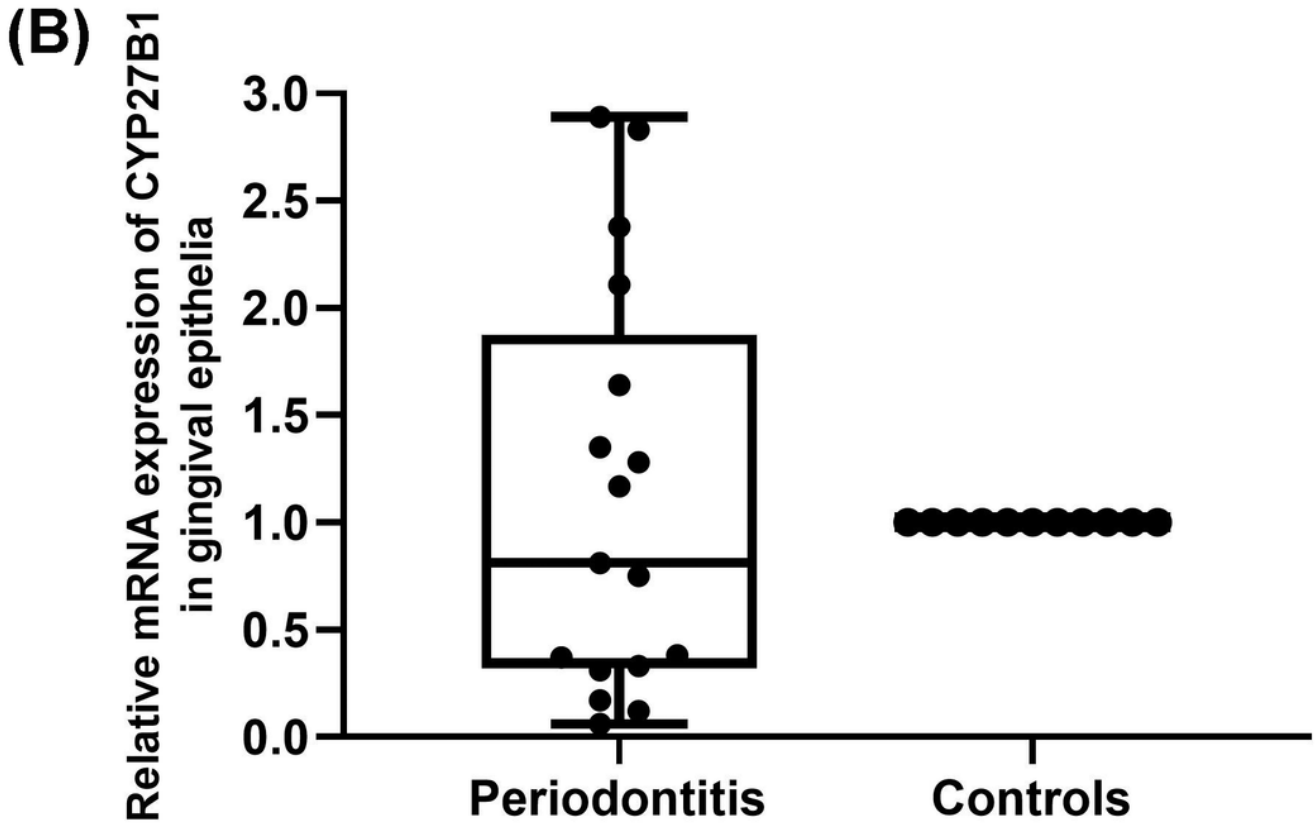

(B)
Relative mRNA expression of CYP27B1 in gingival epithelia
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Periodontitis
Controls

Figure 2

Fig.2 Detection of CYP27B1 in gingiva by immunohistochemistry

Panels (A)–(C) and (D)–(F) show immunohistochemical staining of CYP27B1 in the gingiva of one patient with periodontitis and one control, respectively. Panels (G)–(I) are negative controls. The black frame indicates the epithelial tissue (Panels B, E, H), while the blue frame shows the connective tissue (Panels C, F, I). The expression of CYP27B1 in hGFs is shown by arrows in Panels C and F.

Magnification: (A), (D) and (G) 20×; all others 180×.

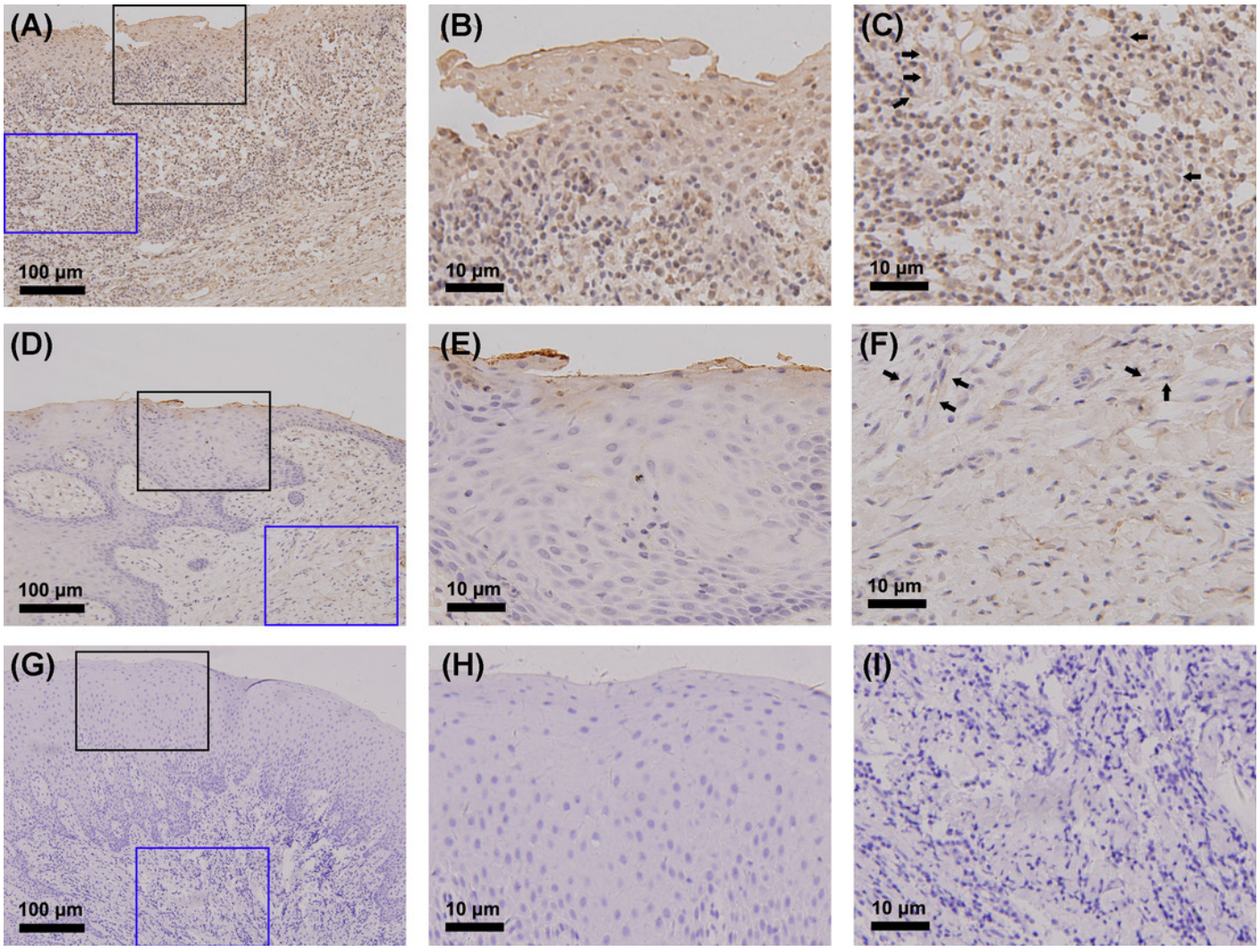


Figure 3

Fig.3 Colocalization of CYP27B1 and vimentin in hGFs

Panels (A)–(C) show immunofluorescence staining of samples from a periodontitis patient, and Panels (D)–(F) show the corresponding results from a control. Panels (A) and (D): CYP27B1; Panels (B) and (E): vimentin; Panels (C) and (F): combination.

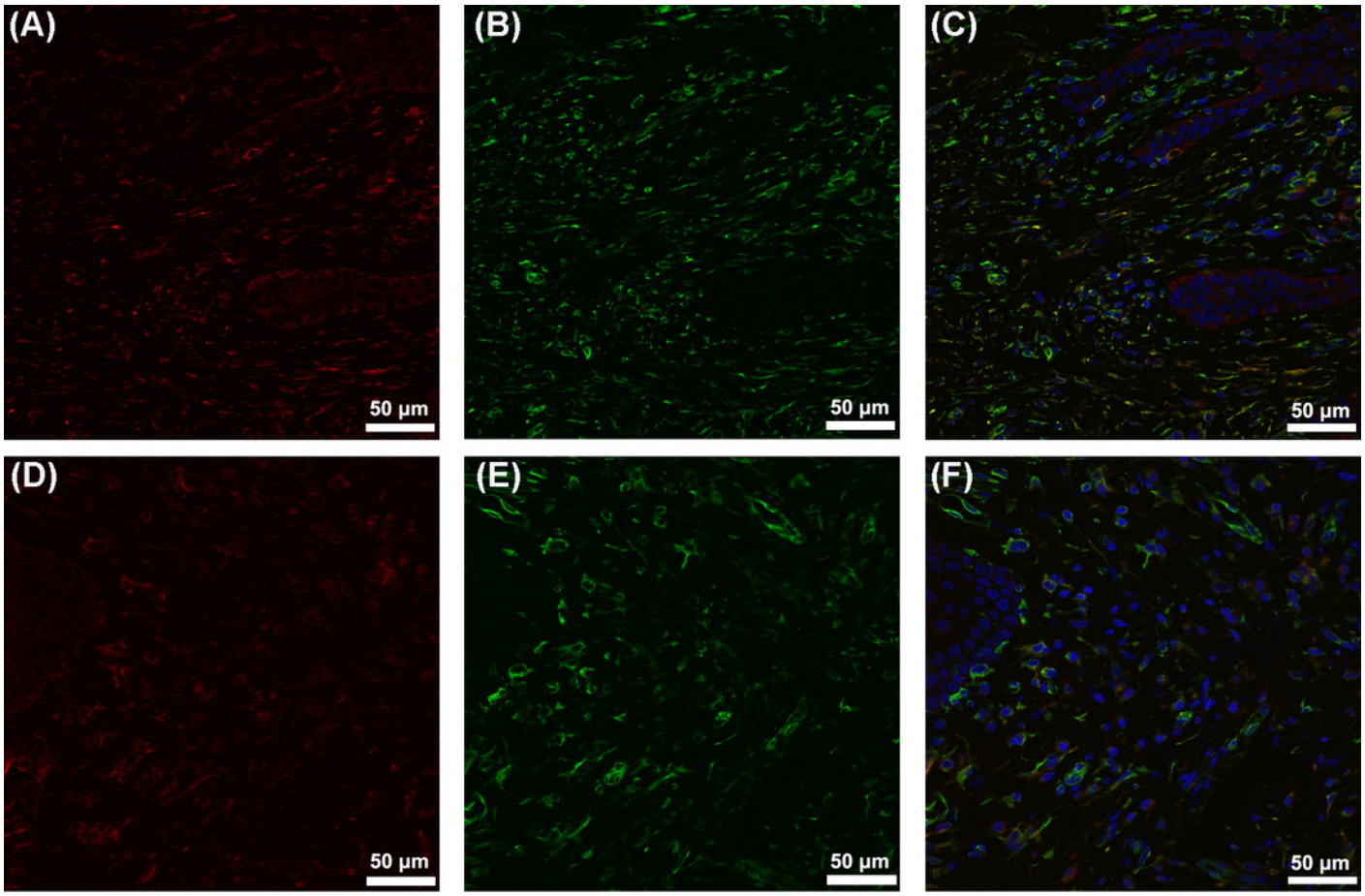


Figure 4

Fig.4 Evaluation of CYP27B1 protein expressions in gingiva

(A) CYP27B1 staining intensities of gingival connective tissues of the diseased group were significantly higher than those of the control group. (B) CYP27B1 staining scores of gingival fibroblasts of the diseased group were significantly higher than those of the control group.

* Compared to the control group, $P < 0.05$.

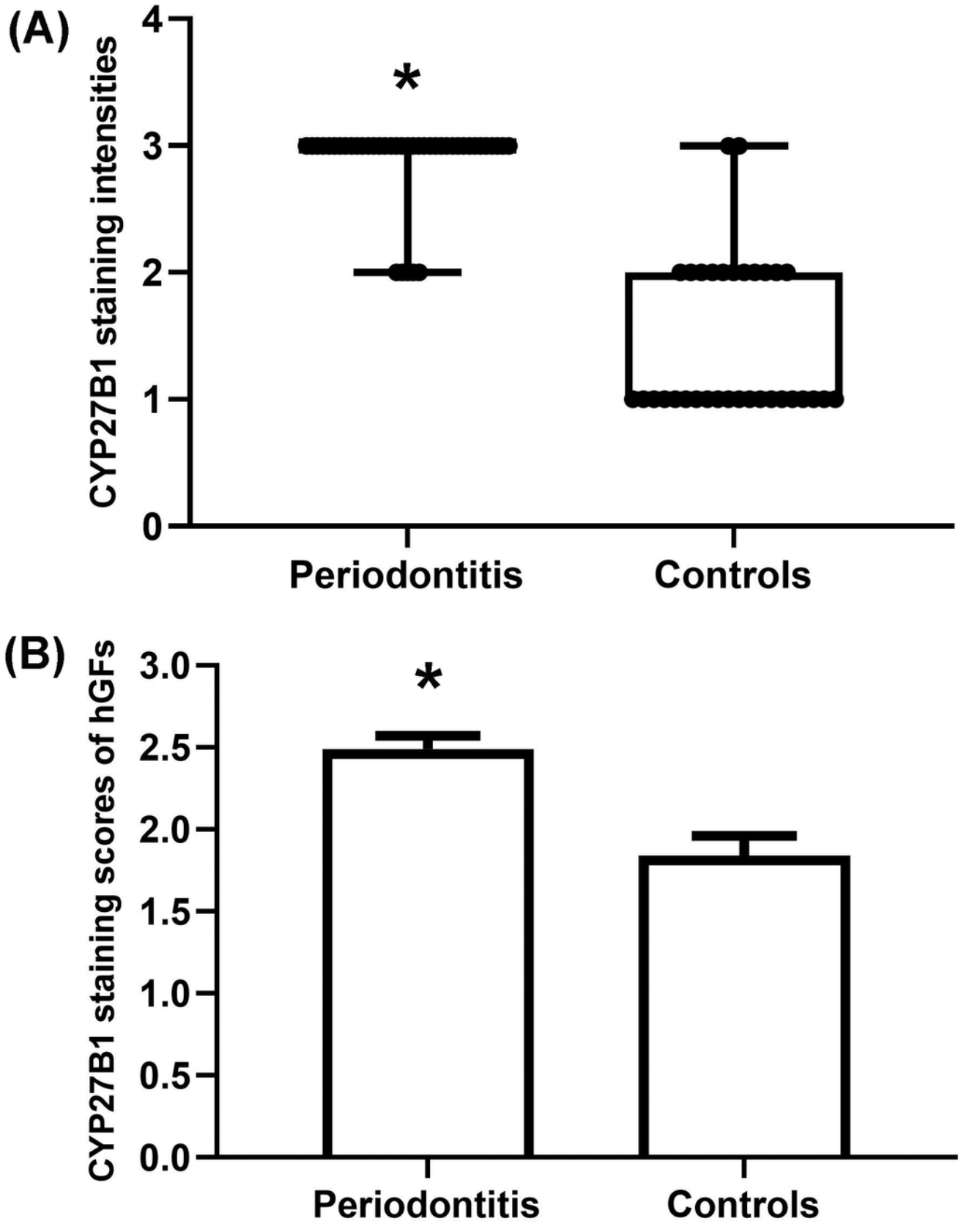

(A)
CYP27B1 staining intensities
4
3
2
1
0
*
Periodontitis
Controls
(B)
CYP27B1 staining scores of hGFs
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Periodontitis
Controls

Table 1(on next page)

Table 1 Demographic data and clinical parameters of the two groups

Table 1 Demographic data and clinical parameters of the two groups

Parameters	Periodontitis (n = 42)	Controls (n = 33)
Age (years)	33.5 ± 7.8	30.0 ± 9.2
Gender (male/female)	20/22	16/17
PD (mm)	7.3 ± 0.4*	1.9 ± 0.5
AL (mm)	5.9 ± 0.6*	0
BI	4*	0 (0 to 0.5)
BOP%	100%*	0

Data are presented as mean ± SD, median (lower to upper quartile), or number of subjects, as indicated

* Compared to the control group ($P < 0.05$)