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SHORT COMMUNICATION

Porphyromonas gingivalis LPS stimulation downregulates DNMT1, DNMT3a, and JMJD3 gene expression levels in human HaCaT keratinocytes

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Abstract
Objective The role of epigenetic regulation in inflammatory diseases such as periodontitis is poorly known. The aim of this study was to assess whether *Porphyromonas gingivalis* lipopolysaccharide (LPS) can modulate gene expression levels of the some enzymes that promote epigenetic events in cultures of the human keratinocytes and gingival fibroblasts. In addition, the same enzymes were evaluated in gingival samples from healthy and periodontitis-affected individuals.
Materials and methods Primary gingival fibroblast and keratinocyte (HaCaT) cultures were treated with medium containing *P. gingivalis* LPS or *P. gingivalis* LPS vehicle for 24 h. After this period, cell viability was assessed by MTT test and total RNA extracted to evaluate gene expression levels of the following enzymes by qRT-PCR: DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a (DNMT3a), histone demethylases Jumonji domain containing 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX). To evaluate gene expression in healthy and periodontitis-affected individuals, total RNA was extracted from biopsies of gingival tissue from healthy and periodontitis sites, and gene expression of DNMT1, DNAMT3a, JMJD3, and UTX was evaluated by qRT-PCR.
Results No significant differences were found in the gene expression analysis between healthy and periodontitis-affected gingival samples. The results showed that LPS downregulated DNMT1 ($p<0.05$), DNMT3a ($p<0.05$), and JMJD3 ($p<0.01$) gene expression in HaCaT cells, but no modulation was observed in gingival fibroblasts.
Conclusion P. gingivalis LPS exposure to human HaCaT keratinocytes downregulates gene expression of the enzymes that promote epigenetic events.
Clinical relevance The advance knowledge about epigenetic modifications caused by periodontopathogens may to possibly led to the development of new periodontal therapies.

Keywords Periodontitis · Epigenetic · *P. gingivalis* LPS · Keratinocytes · Gingival fibroblasts

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Introduction

Periodontitis is a disease that affects up to 20 % of the adult population in industrialized countries [1]. From a pathological point of view, periodontitis can be defined as the presence of gingival inflammation that promotes a pathological detachment of collagen fibers from the cementum and the apical migration of junctional epithelium. Inflammatory events associated with connective tissue attachment loss also lead to the resorption of coronal portions of tooth supporting alveolar bone [2, 3].

Periodontitis is initiated by specific bacteria within plaque biofilm and progresses due to abnormal immune responses by local cells, such as oral keratinocytes, gingival fibroblasts, periodontal fibroblasts, and macrophages to these bacteria [4–7]. Although a number of gram-negative

anaerobic bacteria have been implicated in the disease process, *Porphyromonas gingivalis* (*P. gingivalis*) is considered a major etiologic agent of periodontitis [7, 8]. This bacterium possesses multiple virulence factors, such as lipopolysaccharide (LPS), fimbriae, gingipains, and hemagglutinins, which are believed to contribute to the initiation and progression of periodontal diseases [7, 9].

Inflammatory responses in periodontitis require the activation of a complex gene expression program that involves the inducible transcription of hundreds of genes whose products restrain microbial colonization, recruit and activate leukocytes, increase vascular permeability, amplify the response, and protect inflammatory and tissue cells from apoptosis [10]. The inflammatory microenvironment observed in periodontitis exhibits high variation in the transcription levels of molecules implicated in immune response and periodontal tissue degradation. Recently, studies have discussed the transcriptional regulations in this microenvironment, and some of them suggested that epigenetic alterations could play an important role during the periodontal disease progression [11–13].

Epigenetics is defined as the study of mitotically and meiotically heritable changes in gene function that are not dependent on DNA sequence [14]. Epigenetic mechanisms are not only related to diseases but are also essential for development, cell differentiation, and protection against viral genomes; they also seem to be critical for the integration of endogenous and environmental signals during the lifetime of a cell or an organism [15]. The molecular basis of epigenetic processes is complex and involves modifications of histones, methylation of DNA, positioning of histone variants, and gene regulation by noncoding RNAs.

DNA methylation transcriptional regulation is carried out by two types of DNA methyltransferases (DNMTs): de novo and maintenance methyltransferases [16, 17]. DNA methylation patterns are established during early development by de novo methyltransferases DNA methyltransferase 3a (DNMT3a) and DNMT3b [17, 18]. Patterns of DNA methylation are propagated with extreme fidelity by the maintenance methyltransferase DNA methyltransferase 1 (DNMT1) [17], which reproduce patterns of methylated and unmethylated CpG sites between cell generations [15]. In addition, changes in DNMT levels already were observed in cells exposed to endotoxin, such as LPS [19, 20].

One of the epigenetic events involving histones modification is methylation. Methylation of histone H3 lysines 4 and 27 is catalyzed by the trithorax and polycomb family of developmental regulators [21]. Di- and trimethylation on H3 lysine 27 (H3K27me2/3) is generally associated with transcriptional repression, whereas trimethylation on lysine 4 (H3K4me3) is associated with transcriptional activation [21–23]. The histone H3 lysine 27 (H3K27) demethylases Jumonji domain containing 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) remove the gene-inactivating H3K27 dimethyl and trimethyl marks and are involved in inducing and/or maintaining gene expression [24].

Although there are many studies investigating epigenetic mechanisms in diseases like cancer [14, 25], the involvement of these mechanisms in inflammatory diseases such as periodontitis is poorly understood. In the present study, we hypothesized that LPS derived from *P. gingivalis*, one of the major pathogenic agents in periodontitis, could modulate gene expression of the same enzymes that promote epigenetic events: DNMT1, DNMT3a, JMJD3, and UTX, in culture of human HaCaT keratinocytes and human gingival fibroblasts. Furthermore, it was hypothesized that inflammation in chronic periodontitis sites could modulate gene expression levels of these same enzymes (DNMT1, DNMT3a, JMJD3, and UTX).

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM) and Hank's salt solution were obtained from Cultilab (São Paulo, Brazil). Heat-inactivated fetal bovine serum (FBS) and trypsin/ethylenediamine tetraacetic acid (EDTA) solution used in all experiments were purchased from LGC Biotecnologia (São Paulo, Brazil). The antibiotics (penicillin/streptomycin/amphotericin B) were purchased from GIBCO (Grand Island, NY, USA). Tissue culture multiwell plates were obtained from TPP® (Switzerland). RNAlater used to stored gingival samples were purchased from Ambion Inc. (Austin, TX, USA). *P. gingivalis* lipopolysaccharide (*P. gingivalis* LPS), extracted from *P. gingivalis* strain ATCC 3327, was obtained from InvivoGen (cat. code: tlrl-pglps, San Diego, CA, USA). For the viability analysis, a MTT assay (CellTiter 96® nonradioactive cell proliferation assay) was purchased from Promega (Madison, WI, USA). For the qRT-PCR assays, TRIzol® reagent, DNase, and SuperScript III first-strand synthesis of the oligo(dT) primer were obtained from Invitrogen (Carlsbad, CA, USA), and Fast SYBR Green Master Mix was purchased from Applied Biosystems (Frederick, MD, USA).

Study population and sample collection

To assess whether periodontitis could modulate the expression of the target genes in this study, 20 patients were selected and divided into two groups. The periodontitis group ($n=10$) was comprised of individuals diagnosed with generalized chronic periodontal disease, and the control group ($n=10$) was comprised of systemically healthy

individuals with no periodontal disease. Our sampled population consisted of 11 women and nine men, aged 21 to 66 years (mean age, 45.1±11.8 years). The samples for the periodontitis group were collected from sites presenting probing depths ≥5 mm with clinical attachment loss ≥4 mm and bleeding on probing which required surgical intervention. Control group samples were collected from sites presenting no bleeding on probing with probing depth ≤3 mm and no clinical attachment loss in patients who had periodontal plastic surgery due to esthetic reasons. All patients from this study signed consent forms approved by the Institutional Review Board of the State University of Campinas, São Paulo, Brazil (123/2010). All subjects underwent anamnesis and clinical periodontal examination. Exclusion criteria included any systemic disorder that would require antibiotic prophylaxis or would affect periodontal condition. All patients were nonsmokers, and female patients were not pregnant, lactating, or using hormonal birth control methods. After the gingival biopsies were obtained, they were rinsed with cold sterile saline solution and stored in a tube containing RNAlater at −70°C.

Primary cell culture

Human gingival tissue biopsies from healthy sites were obtained from subjects undergoing periodontal surgery for non-disease-related reasons (e.g., esthetics). The donor patients had the same characteristics as described for individuals in the control group in mentioned in "Study Population and Sample Collection." The patients signed consent forms approved by the Institutional Review Board of the State University of Campinas, São Paulo, Brazil (123/2010).

Biopsies from connective tissue of human gingival tissue were washed three times in Hank's salt solution containing penicillin (100 units/mL)/streptomycin (100 μg/mL)/amphotericin B 5 μg/mL, and then incubated in DMEM containing 10 % FBS and penicillin/streptomycin/amphotericin B up to 4 h at room temperature. The specimens were minced into small pieces (explants with ~3×3×3 mm) and placed in a culture dish at 37°C in a high-humidity and 5 % CO_2 atmosphere. When the cells growing out from explants reached confluence, the pieces of gingival tissue were removed. The cells (gingival fibroblasts) were then trypsinized using a solution of 0.05 % trypsin/0.02 % EDTA in phosphate-buffered saline and placed in a second culture dish. The experiment was performed using cells between the third and sixth passages.

LPS treatment

Immortalized human keratinocytes (HaCaT) were kindly provided by Professor Dr. Ricardo Coletta from the Department of Oral Diagnosis of the Piracicaba Dental School, São Paulo, Brazil. Human gingival fibroblast (GF) were cultured in DMEM supplemented with 10 % heat-inactivated FBS and penicillin (100 units/mL)/streptomycin (100 μg/mL) at 37°C in a high-humidity and 5 % CO_2 atmosphere. At harvest, the cells were plated at a concentration of 2×10^5/mL in multiwell plates and cultured until they reached a confluent state (~48 h to HaCaT cells and 72 h to GF). Confluent cells were exposed to 500 ng/mL of the *P. gingivalis* LPS diluted in sterile water for 24 h at 37°C in a high-humidity and 5 % CO_2 atmosphere, and then cell viability and gene expression were evaluated. During the experimental period, cells were cultured with DMEM supplemented with 2 % FBS. Cultures treated with LPS vehicle (deionized water used to diluted *P. gingivalis* LPS) served as controls.

Viability assay

Cell viability was assessed by MTT reduction to formazan crystals by mitochondrial enzyme reductases using a MTT biochemical assay in 96-well plates (n=5). Initially, it was added at the experimental medium of 15 μL of MTT solution and incubated for 4 h at 37°C in 5 % CO_2 atmosphere. Control wells without cells containing experimental medium were incubated in parallel with test samples to measure the absorbance background. Afterwards, 100 μL of solubilization/stop solution was added to solubilize the formazan product and incubated for 1 h at 37°C in 5 % CO_2. Finally, the multiwell plate was mixed until complete salt crystal dissolution and absorbance was measured in an ELISA reader (Molecular Devices, CA, USA) using the software VersaMax (test wavelength 570 nm, reference wavelength 630 nm).

Gene expression analysis by qRT-PCR

Reverse transcription followed by qPCR was utilized in order to evaluate DNMT1, DNMT3a, JMJD3, and UTX gene expression in two different conditions: (1) gingival samples from healthy or periodontitis individuals and (2) HaCaT cells and human gingival fibroblasts treated with *P. gingivalis* LPS (or vehicle). After sample collection (biopsies) or cell treatment (HaCaT and GF), as previously described, total RNA was extracted using TRIzol® reagent, following the manufacturer's recommendation. RNA quantification and purity were measured by photometric measurement using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and RNA quality was assessed by electrophoresis on a denaturing 2 % agarose gel. One microgram of total highly purified RNA was treated with DNase, and then 0.5 μg of RNA was used for cDNA synthesis. The reaction was carried out using the SuperScript III first-strand synthesis of the oligo(dT) primer following the manufacturer's recommendations.

Real-time PCR was conducted in the StepOnePlus (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using JumpStart SYBR Green Taq ReadyMix™. To record PCR reactions in real time, the following primer sequences were used: DNMT1 F: 5'-CTGAGGCCTTCACGTTCA-3', R: 5'-CTCGCTGGAGTGGACTTGT-3'; DNMT3a F: 5'-CAG CGTCACACAGAAGCATATCC-3', R: 5'-GGTCCTCAC TTTGCTGAACTTGG-3'; JMJD3 F: 5'-AGCTGGCCCTG GAACGATA-3', R: 5'-GGCCCTGGTAAGCGATTT-3'; DNMT3a F:5'-TACAAATCCGAACAACCC-3', R: 5'-TG AGGAGGCCTGGTACTGT-3'; and GAPDH F: 5'-GAAGG TGAAGGTCGGAGTC-3', R: 5'-GAAGATGGTGATGGGA TTTC-3'. The threshold was set above the nontemplate control background and within the linear phase of the target gene amplification to calculate the cycle number at which the transcript was detected, denoted as the crossing point. Target genes' expressions were normalized by the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

All results are expressed as mean ± standard deviation (SD). Statistical analysis was completed using unpaired Student's *t* test (two-tailed). Statistical significance limit was set at 5 % ($p<0.05$).

Results

MTT assay was used to determinate the effect of LPS treatment on HaCaT and gingival fibroblast viability, and the results showed that 500 ng/mL of the LPS did not affect viability of both cell types (Fig. 1).

Keratinocytes (HaCaT) and GF were treated with LPS from *P. gingivalis* in order to evaluate if this stimulation could modulate DNMT1, DNMT3a, JMJD3, and UTX gene expression. The exposure of HaCaT cells to LPS for 24 h led to a decrease in DNMT1 ($p<0.05$), DNMT3a ($p<0.05$), and JMJD3 ($p<0.01$) gene expression levels (Figs. 2 and 3), when compared to HaCaT cells exposed to LPS vehicle (deionized water). The UTX gene expression in HaCaT cells was not modulated by LPS stimulation ($p>0.05$). Gingival fibroblasts were exposed to LPS or LPS vehicle for 24 h, and the gene expression analysis demonstrated that LPS stimulation did not change the mRNA expression levels of DNMT1, DNMT3a, JMJD3, and UTX when compared to control (vehicle) (Figs. 2 and 3).

Total RNA was extracted from gingival biopsies of the individuals with or without periodontitis, and the gene expression analysis demonstrated that periodontitis did not change the gene expression levels of the DNMT1, DNMT3a, JMJD3, and UTX when compared to healthy samples (Figs. 2 and 3).

Discussion

Cellular activation, proliferation, and survival in chronic inflammatory diseases are regulated not only by engagement of signal transduction pathways that modulate transcription factors required for these processes but also by epigenetic regulation of transcription factor access to gene promoter regions [26]. Thus, studies on rheumatoid arthritis, systemic sclerosis, systemic lupus erythematous, and periodontitis have reported an epigenetic regulation of genes related to cell proliferation, inflammatory responses, and tissue remodeling [12, 26–28]. Our experiments demonstrated, for the first time, that LPS from *P. gingivalis*, which is considered one of the major etiological agents of periodontitis, can modulate gene expression of enzymes that control some epigenetic events.

The gingival epithelium is the first physical barrier to periodontopathogens, such as *P. gingivalis* LPS, before the beginning of the process that leads to periodontal inflammation [29].

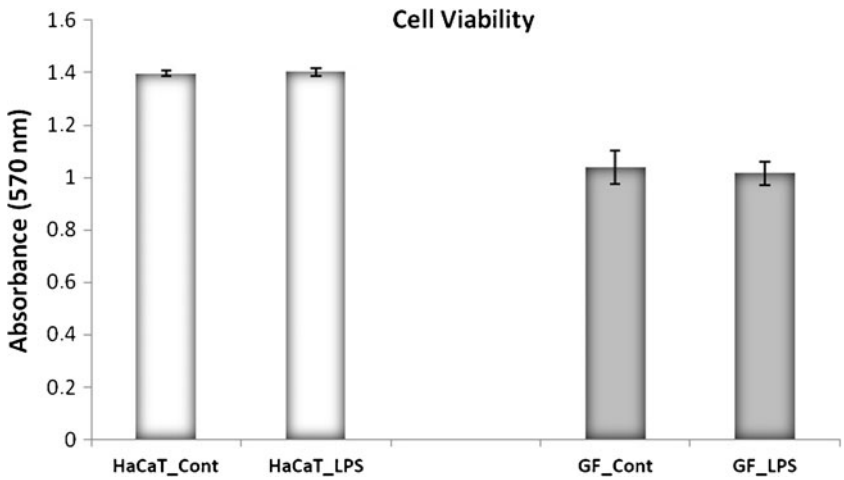

Fig. 1 The figure represents the mean ± SD of the values obtained for cell viability after exposure of human keratinocytes (HaCaT) and human gingival fibroblasts (GF) cells to 500 ng/mL of the *P. gingivalis* LPS (HaCaT_LPS and GF_LPS) or vehicle (HaCaT_Cont and GF_Cont) for 24 h. No significant differences on the HaCaT or GF cells viability were found by LPS stimulation ($n=5$/group)

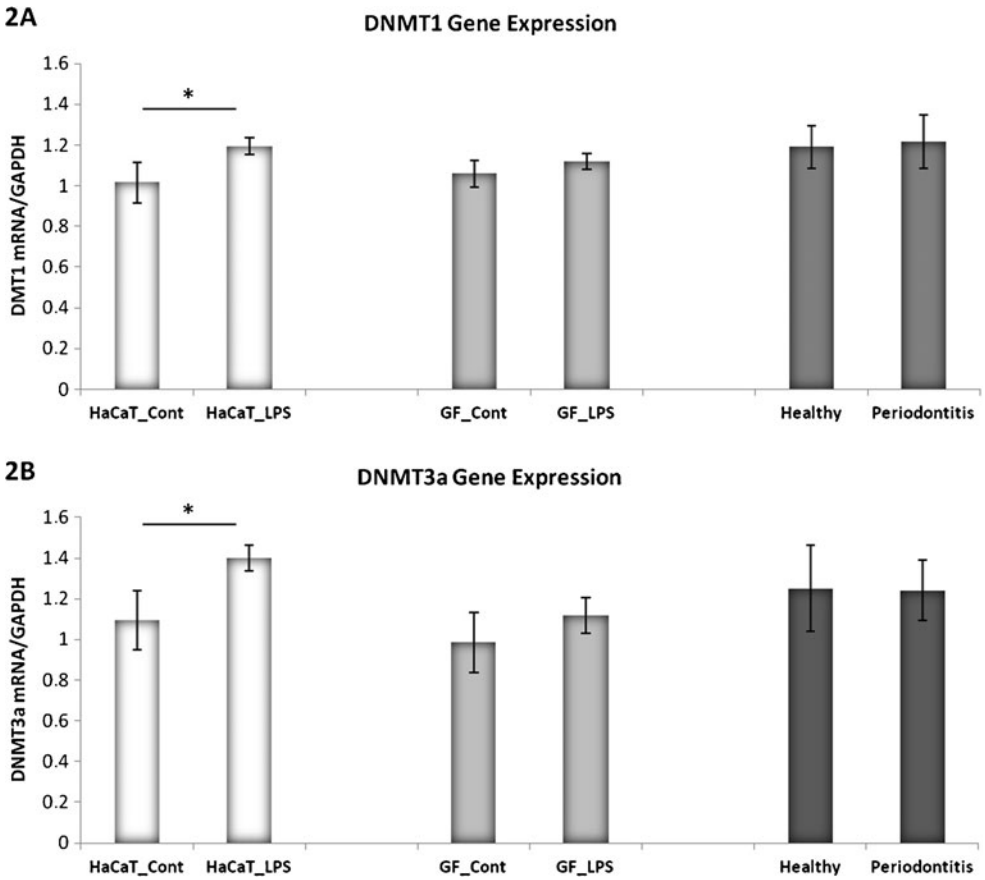

Fig. 2 The figures represent mean ± SD of the values obtained for DNA methytransferase 1 (DNMT1) and DNMT3a gene expression analysis. The results showed that the treatment of HaCaT cells with 500 ng/mL of the *P. gingivalis* LPS (HaCaT_LPS) for 24 h reduces DNMT1 (**a**) and DNMT3a (**b**) gene expression levels when compared to control (HaCaT_Cont, vehicle treatment). LPS treatment in gingival fibroblasts (GF) did not change mRNA transcription levels for DNMT1 (**a**) or DNMT3a (**b**) when compared to control. Samples from healthy or periodontitis individuals showed similar gene expression levels of DNMT1 (**a**) and DNMT3a (**b**). For both **a** and **b**, the data are expressed as the ratio of target genes to reference gene (GAPDH). Gene expression means obtained for vehicle groups was assumed as 100 % ± SD in each experimental condition. *$p<0.05$, means are statistically different. $n=3$/groups to HaCaT and GF; $n=10$/group to healthy and periodontitis groups

LPS is an essential macromolecule that comprises the outer surface of gram-negative bacteria, and when this molecule is recognized by the host as a foreign molecule, it elicits an immune response that is designed to eliminate the bacterial invasion. The recognition of the *P. gingivalis* LPS by cells from the gingival tissue has been shown to depend on the binding of this molecule to toll-like receptor (TLR) 2 and TLR4 [30–32].

Although it has been reported that human gingival fibroblasts respond to *P. gingivalis* LPS stimulation [33], the results from our study showed that only keratinocytes (HaCaT) responded to *P. gingivalis* LPS in the evaluated parameters. Epithelial cells, which express TLRs, may function as nonprofessional inflammatory cells and help professional cells of the innate and adaptive immune system to clear the bacterial infection. The activation of TLRs from oral epithelial cells promotes the release of proinflammatory and chemotactic cytokines, matrix-degrading enzymes, and prostaglandins [5, 32–35]. Therefore, oral epithelial cells can actively participate in periodontal inflammation.

Upon TLR engagement, LPS triggers an intracellular signaling cascade which involves, among other factors, the nuclear transactivation of nuclear factor-kappa B (NF-κB) [31, 32]. NF-κB is a family of transcription factors required for the induction of the most important classes of inflammatory genes, including genes involved in periodontitis progression. Both recruitment of NF-κB to target promoters and NF-κB-induced transcriptional genes can be modulated through chromatin modification [36]. In our study, it was found that the gene expression of the histone demethylase JMJD3, which is directly regulated by NF-κB [37], was decreased in keratinocytes treated with *P. gingivalis* LPS.

The expression of the JMJD3 is quickly and strongly induced in macrophages exposed to LPS; in addition, JMJD3 is involved in gene silencing during inflammation [37]. Since JMJD3 binds to target genes and regulates their

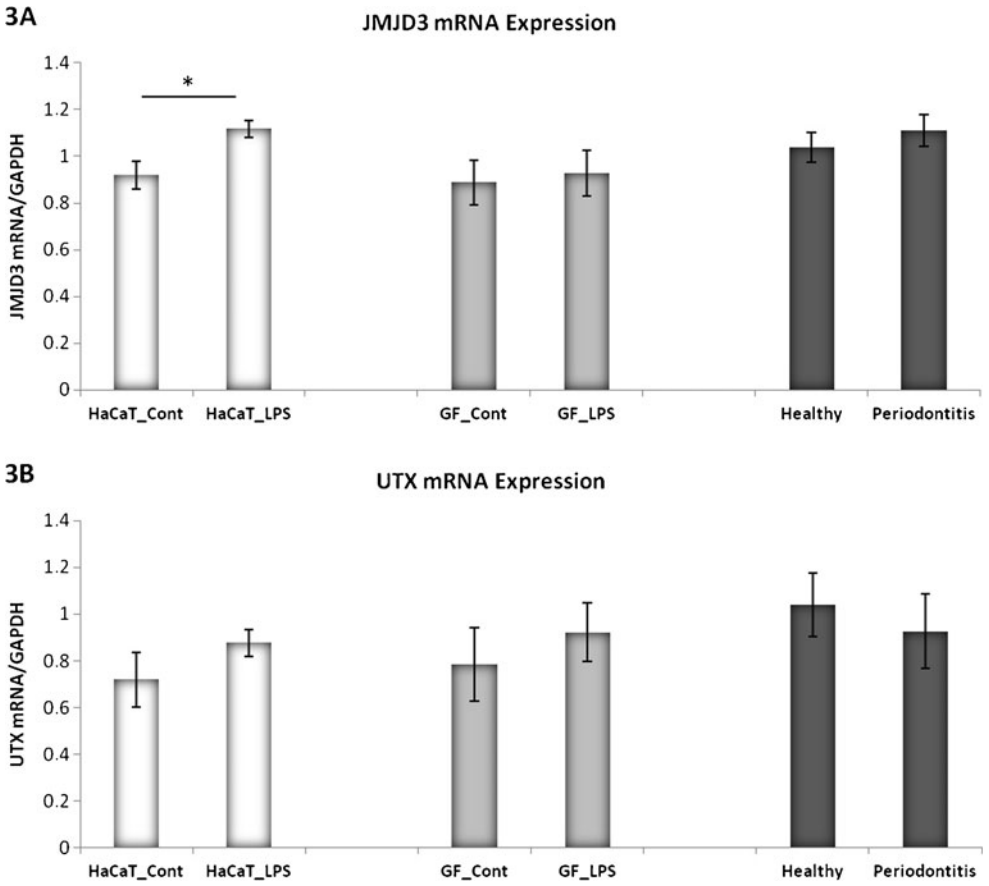

Fig. 3 The figures represent mean ± SD of the values obtained for JMJD3 and UTX gene expression analysis. The results showed that treatment of HaCaT cells with 500 ng/mL of *P. gingivalis* LPS (HaCaT_LPS) for 24 h reduced JMJD3 (**a**) gene expression levels when compared to control (HaCaT_Cont, vehicle treatment). LPS treatment of HaCaT did not change mRNA transcription levels of UTX (**b**). Gene expression levels of JMJD3 (**a**) and UTX (**b**) were not modulated by LPS in gingival fibroblasts (GF). Samples from healthy individuals or those with periodontitis showed similar gene expression levels of JMJD3 (**a**) and UTX (**b**). For both **a** and **b**, the data are expressed as the ratio of target genes to reference gene (GAPDH). Gene expression means obtained for vehicle groups was assumed as 100 % ± SD in each experimental condition. *$p<0.01$, means are statistically different. ($n=3$/groups to HaCaT and GF; $n=10$/group to healthy and periodontitis groups)

H3K27me3 levels and transcriptional activity, the decrease of the gene expression levels of JMJD3 found in our study could lead to the modification of the transcription levels of the inflammatory genes activated by LPS.

It has generally been accepted that increased methylation (hypermethylation) in the gene promoter region is associated with a decrease in gene expression, while hypomethylation pattern is closely associated with transcriptional upregulation [11, 38]. The change in methylation status in CpG islands of DNA, which are regions of the genome that contain a high percentage of CpG dinucleotides, is profoundly associated with diseases such as developmental abnormalities, cancer, and chronic inflammatory states [11, 17, 39].

We found that the gene expression of DNMT3a and DNMT1, enzymes which promote and maintain DNA methylation, was downregulated when keratinocytes were exposed to *P. gingivalis* LPS. Our results corroborated with the findings from Yin and Chung [37], which showed that *P. gingivalis* (whole bacteria) can cause a decrease in DNMT1 gene expression in oral epithelial cells. In addition, these authors also demonstrated that *P. gingivalis* may modulate DNA methylation status of the genes involved in the pathogenesis of periodontitis.

Our results did not demonstrate changes in the gene expression of the enzymes evaluated in samples from biopsies (healthy × periodontitis). The different cell types found in tissues from sites affected by periodontitis, such as epithelial cells, fibroblasts, and inflammatory cells, may have a specific epigenetic profile and make it difficult to assess the real role of each cell in the epigenetic events in that microenvironment.

In summary, this study demonstrated that LPS from *P. gingivalis* may modulate the gene expression of important enzymes involved in epigenetic control in human HaCaT keratinocytes. Further investigations should be done, in an attempt to understand the role of epigenetic regulation in the pathogenesis of periodontitis. The advanced knowledge in this area may

allow for the development of epigenetic therapies, especially in complex multifactorial diseases such as periodontitis.

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Conflicts of interest The authors declare that they have no conflict of interest.

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