UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GLÁUCIA DE CAMARGO PEREIRA

ESTUDO DA PERIODONTITE CRÔNICA E DA EXPOSIÇÃO DE LPS DE P. GINGIVALIS A FIBROBLASTOS GENGIVAIS E QUERATINÓCITOS, NA MODULAÇÃO DA EXPRESSÃO DE GENES REGULADORES DE EVENTOS EPIGENÉTICOS

Dissertação de Mestrado apresentada à Faculdade de Odontologia de Piracicaba da UNICAMP para obtenção do Título de Mestre em Biologia Buco-Dental, área de concentração Histologia e Embriologia.

Orientador: Prof. Dr. Marcelo Rocha Marques

Este exemplar corresponde à versão final da dissertação defendida pela aluna e, orientada pelo Prof. Dr. Marcelo Rocha Marques

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Prof. Dr. Marcelo Rocha Marques

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Dedicatória

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"É melhor tentar e falhar,
que preocupar-se e ver a vida passar;
é melhor tentar, ainda que em vão,
que sentar-se fazendo nada até o final.
Eu prefiro na chuva caminhar,
que em dias tristes em casa me esconder.
Prefiro ser feliz, embora louco,
que em conformidade viver ..."

Martin Luther King

RESUMO

A periodontite crônica é uma doença inflamatória que leva à perda de inserção de elementos dentários, e é desencadeada e mantida por um biofilme subgengival periodontopatogênico. A presença de alguns tipos de lipopolissacarídeos (LPS), derivados de bactérias no sítio periodontal doente, pode iniciar uma sinalização por meio das células do tecido gengival, que culminará com um microambiente com diferentes células do sistema imune e com uma alteração no padrão de expressão de citocinas inflamatórias. Já foi evidenciado que no tecido gengival de pacientes com periodontite crônica, genes que codificam receptores celulares para o LPS, podem sofrer alterações epigenéticas. O objetivo deste estudo foi avaliar se a periodontite crônica e o LPS bacteriano derivado de *P. gingivalis* podem modular a expressão gênica de alguns fatores reguladores de eventos epigenéticos. Biópsias de tecido gengival inflamado e sem inflamação foram coleados de pacientes com periodontite crônica e de pacientes saudáveis respectivamente, o RNA total foi extraído e a expressão dos genes DNMT1 (DNA metiltransferase 1), DNAMT3a (DNA metiltransferase 3a), histona demetilase JMJD3 e histona demetilase UTX foram analisadas por meio de RT-PCR quantitativo. Fibroblastos gengivais humanos derivados de cultura primária, e queratinócitos (HaCaT) foram expostos a LPS de *P. gingivalis* ou ao veículo do LPS, e foram avaliadas a viabilidade celular por meio do teste MTT e a expressão gênica de DNMT1, DNMT3a, JMJD3 e UTX por meio de RT-PCR quantitativo. As análises dos resultados demonstraram que nem a periodontite e nem o LPS exposto a fibroblastos gengivais foram capazes de modular a expressão dos genes estudados. Contudo, o LPS promoveu a diminuição da expressão de DNMT1, DNMT3a e JMJD3 nas células HaCaT. Pode-se concluir que LPS derivado *P. gingivalis* pode modular, em queratinócitos, a expressão gênica de algumas enzimas promotoras de eventos epigenéticos.

Palavras chave: Expressão gênica, DNA (Citosina-5-) metiltransferase, Epigênese genética.

ABSTRACT

The aim of this study was to assess whether *P. gingivalis* LPS can modulate, in culture of the human keratinocytes and human gingival fibroblasts, gene expression levels of the some enzymes that promote epigenetic events. In addition, the same enzymes were evaluated in sample from healthy and periodontitis affected individuals. Primary gingival fibroblast culture and keratinocytes (HaCaT) were treated with medium containing *P. gingivalis* LPS or *P. gingivalis* LPS vehicle for 24hs. After this period, cell viability were assessed by MTT test, and total RNA were extracted to evaluate gene expression levels of the enzymes: DNMT1 (DNA methyltransferase 1), DNMT3a (DNA methyltransferase 3a), histone demethylases JMJD3 and UTX, by qRT-PCR. To evaluate the gene expression in healthy and periodontitis affected individuals, total RNA was extracted from biopsies of gingival tissue from sites with (periodontitis) or without periodontitis (healthy), and gene expression of DNMT1, DNAMT3a, JMJD3 and UTX were evaluated by qRT-PCR. No significant differences were found in the gene expression analysis between healthy gingival tissues and gingival tissue from periodontitis sites. 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 found to gingival fibroblasts. *P. gingivalis* LPS exposure to keratinocytes, downregulates gene expression of the enzymes that promote epigenetic events.

Key Words: Gene expression, DNA (Cytosine-5-)-methyltransferase, Epigenesis, genetic

LISTA DE ABREVIATURAS E SIGLAS

CpG - citosina/ ligação fosfodiéster/ guanina

DNA - ácido desoxirribonucleico

DNMT - DNA metiltransferase

DNMT 1 - DNA metiltransferase 1

DNMT 2 - DNA metiltransferase 2

DNMT 3a - DNA metiltransferase 3a

DNMT 3b - DNA metiltransferase 3b

JMJD3 - Jumonji Domain Containing 3

HMTs - histone methyltansferase

H3K24 - H3 lysine 24

H3K27 (H3 lysine 27)

IL-8 - interleucina 8

IL – interleucina

IL-1a – interleucina 1a

IL-1β interleucina 1 beta

IL-6 – interleucina 6

LPS – lipopolissacarídeo

MLL2-4 - mixed lineage leukemia

P. gingivalis - *Porphyromonas gingivalis*

UTX - Ubiquitously Transcribed Tetratricopeptide Repeat, X chromosome

SUMÁRIO

INTRODUÇÃO

Até 90% da população mundial pode ser afetada pelas doenças periodontais. A gengivite, a forma mais suave de doença periodontal, é causada pelo acúmulo de biofilme bacteriano sobre a superfície dental (Pihlstrom BL *et al.* 2005). A periodontite, a forma mais severa da doença periodontal, é uma doença inflamatória crônica, caracterizada pela destruição progressiva dos tecidos de suporte dentário,o periodonto (Kraus D. *et al.* 2012). É um processo complexo e multifatorial, no qual as interações entre os componentes da placa bacteriana subgengival e os mecanismos de defesa do hospedeiro determinam o início e o desenvolvimento da doença (Nanci & Bosshardt, 2006). É o resultante da resposta imunoinflamatória do hospedeiro ao desafio bacteriano (Gomez et al., 2009, Kinane & Hart, 2003) gerando alterações na homeostase do tecido conjuntivo e tecido ósseo, sendo influenciada por fatores de risco que agravam a doença (por ex. tabaco e higiene bucal).

A doença é causada por bactérias patógenas incorporadas no biofilme sobre a superfície do dente ocasionando a proliferação epitelial em combinação com a formação da bolsa periodontal, o aumento da mobilidade do dente e até mesmo a perda do elemento dental (Kraus D. *et al.* 2012). Participam desse processo bactérias gram-negativas, com níveis de prevalência elevados (Nanci & Bosshardt, 2006). O início e a manutenção da periodontite devem-se à contínua presença de biofilme, com altos níveis (concentrações altas) de substratos para o desenvolvimento de centenas de espécimes bacterianos (Tonetti & Claffey, 2005). A microbiota bacteriana presente na cavidade oral é muito importante para o periodonto saudável ou doente (Darveau 1997, Tonetti 1998, Tonetti & Claffey, 2005). Uma das causas mais significativas do desenvolvimento da doença periodontal é o desequilíbrio das concentrações bacterianas Gram-negativas presentes no biofilme bacteriano (Kinane & Hart, 2003). Dentre estas, a *Porphyromonas gingivalis* (*P. gingivalis*) é uma das mais importantes para o

desenvolvimento da periodontite do adulto (Socransky & Haffajee, 1992) pela sua alta capacidade de penetração, devido à presença de inúmeras vesículas contendo lipopolissacarídeos (LPS) (Darveau *et al.*, 2004).

O LPS é uma macromolécula importante sobre a superfície externa da membrana do *P. gingivalis* tendo por função se ligar aos receptores TLR2 e TLR4 (Jain & Darveu, 2010 ; Kocgozlu *et al.* 2009). Após seu reconhecimento pelo receptor uma cascata de sinalização intracelular é desencadeada envolvendo a ativação de NFkB (Jain & Darveu, 2010).

A primeira barreira enfrentada pelas bactérias periodontopatógenas é o epitélio gengival, sendo este uma barreira física (Kinane et al. 2008). Foi demonstrado que LPS de P. gingivalis induz a expressão e liberação de citocinas pró-inflamatórias em células epiteliais bucais, o que sublinha o papel negativo do patógeno em doenças periodontais (Kraus D. et al. 2012).

Os fibroblastos gengivais, através da secreção de colágeno, desempenham um papel importante na remodelação dos tecidos moles periodontais, e são os alvos preferenciais dos LPS. Estas células também funcionam como reguladores de citocinas em tecidos periodontais sendo responsáveis pela produção de diversos tipos quando estimuladas por citocinas inflamatórias ou componentes de células bacterianas (Wang & Ohura, 2002). A periodontite resulta em uma destruição progressiva dos tecidos de proteção e suporte dentário, especificamente a gengiva, o ligamento periodontal e o osso alveolar (Nanci & Bosshardt, 2006).

Estudos recentes evidenciam a importância da epigenética nos mecanismos regulatórios do controle da resposta inflamatória (Wilson, 2008). Evidências sugerem que os eventos epigenéticos em genes de citocinas podem ser importantes na compreensão da patogênese de doenças inflamatórias em que a expressão de citosinas já é regulamentada, como por exemplo, a periodontite (Offenbacher *et al.,* 2008, Oliveira *et al.*, 2009). Considerando-se a natureza complexa e multifatorial da doença periodontal, é possível assumir que eventos

epigenéticos podem contribuir para o desenvolvimento e fenótipo da doença (Offenbacher *et al.,* 2008).

O termo epigenética surgiu na metade do século 20 após estudos correlacionando bases genéticas e embriológicas. Conrad Waddington (1942) foi o pioneiro ao notar uma relação entre elas e acreditou que deveriam de alguma forma, ser estudadas em conjunto (Oliveira NFP, 2009). Surgiu então a epigenética, nome derivado do termo grego *epigenesis* que descreve uma teoria da biologia do desenvolvimento que propõe que embriões em estágios iniciais são indiferenciados (Oliveira NFP, 2009). Nos estágios iniciais de formação do embrião, onde há exclusivamente a participação de células tronco indiferenciadas, o código genético já está determinado. Para o desenvolvimento, o embrião necessita de alteração de expressão gênica para ocorrer a diferenciação celular e a completa formação do feto. Isto ocorre através do ligamento e desligamento de alguns genes durante a divisão celular, mecanismo denominado como alteração epigenética (Oliveira NFP, 2009).

Nas duas últimas décadas a epigenética e principalmente a metilação do DNA vem sendo o principal foco dos pesquisadores, os quais têm demonstrado o papel importante da epigenética na embriogênese, desevolvimento e também em doenças como câncer e osteoartrites. Fenômenos epigenéticos podem ser observados durante o desenvolvimento das células, e caracteriza-se por uma mudança na expressão gênica sem alteração do código genético (Adcock *et al*. 2007; Baylin, 2005). Em células humanas ocorre a desacetilação das histonas e a metilação do DNA (Shaw, 2009). Durante a metilação do DNA ocorre o acréscimo de um grupo metil a uma citosina em uma ilha CpG, enquanto na desacetilação de histona ocorre a remoção de um grupo acetil, alterando assim a forma de empacotamento da histona (Johnson *et al*., 2008).

A falta de conhecimento sobre a metilação de CpG em sítios específicos de promotores de células somáticas dificulta o entendimento completo de como as mudanças na transcrição do DNA influencia o desenvolvimento normal ou causa a doença. O padrão de metilação do DNA varia entre diferentes

células e tecidos, ou mesmo entre células dentro de um mesmo tecido, ao contrário do código genético que é idêntico em todas as células somáticas. A metilação do DNA também pode ser relacionada à presença crônica de uma inflamação e infecção bacteriana (Stenvenkel *et al*., 2007, Bobtesis *et al.,* 2007). Diversas doenças infecciosas também têm o seu prognóstico determinado pelo processo epigenético que é modulado por citocinas (Sanders 2006; Reiner 2005).

A metilação do DNA é um tipo de modificação química do DNA que pode ser herdada e subsequentemente removida sem alteração da sequência original do DNA. Como tal, a metilação é parte do código epigenético e também o mais bem caracterizado mecanismo. Um exemplo é a adição de um grupo metil (CH_3) na posição C5 da citosina que precede uma guanina no anel de pirimidina dentro das regiões CpG, neste caso com o específico efeito de reduzir a expressão gênica (Johnson *et al.,* 2007); podendo comprometer muitas funções celulares como proliferação e reparação (Adcock *et al.* 2007)

Diversas funções do genoma humano são controladas pela metilação do DNA, como por exemplo: recombinação durante meiose e mitose, controle da replicação, controle de DNAs “parasitas” que se inserem no genoma humano (ex.: DNA viral), estabilização e manutenção da expressão gênica, regulação da diferenciação celular, inativação do cromossomo X, sendo essencial durante a morfogênese para que ocorra desenvolvimento normal (Wolffe & Matzke, 1999; Suter *et al.,*2004). A expressão do gene pode sofrer alterações graças à variação dos padrões de metilação do DNA, particularmente na região promotora de genes (Gopisetty *et al.,* 2006).

A metilação consiste em uma modificação covalente do DNA na qual um grupamento metil (CH_3) é transferido da S-adenosilmetionina para o carbono 5 de uma citosina que geralmente precede uma guanina (dinucleotídeo CpG), pela ação de uma família de enzimas que recebe o nome de DNA metiltransferase (DNMT) (Oliveira NFP, 2009). A manutenção da metilação é feita pela DNMT1 (dnametiltransferase 1) em fitas hemimetiladas do DNA, e fitas dos processos de metilação *de novo*, onde há a adição de um grupo metil em sítios sem a presença

de metilação prévia, como as DNMT2, DNMT3a e DNMT3b (Bestor, 2000; Johnson, 2007), as quais são importantes no estabelecimento embrionário dos padrões de metilação (Okano *et al,* 1999). A metionina, seguida do folato, colina e vitamina B12 presentes na alimentação são os principais doadores de radicais metil (Waterland & Jirtle, 2003; Waterland, 2006; Zeisel, 2009).

Áreas ricas em CpG são chamadas de ilhas CpG, compostas por 200pb com aproximadamente 60% de CG, encontradas próximas a regiões promotoras dos genes. A metilação do DNA ocorre quase exclusivamente em dinucleotídeos CpG de células diferenciadas e tem uma importante função na regulação da expressão gênica e no silenciamento de elementos repetitivos no genoma (Feinberg & Tycko, 2004), sendo ela randômica ou sítio específica, sendo frequente em genomas de eucariotos (Poole *et al.,* 2001).

Metilação do DNA é comumente associada com altos níveis ou funções alteradas de DNMTs, enzimas que dão início ao processo de metilação na posição C5 de citosinas de dinucleotídeos CpG. Além disso, modificações de histonas, particularmente metilação e acetilação, podem também estar envolvidas em silenciamento transcricional de vários genes em cânceres (Nandakumar *et al.,* 2011).

A expressão significativa das metiltransferases de DNA, DNMT1 enzima responsável pela manutenção do padrão de metilação e DNMT3a de DNA metiltransferase *de novo*, estão funcionando normalmente em células de divisão rápida para estabelecer e/ou manter os padrões de metilação do DNA. Por exemplo, durante a proliferação celular, DNMT1 localiza os focos de replicação e em primeiro lugar metila o DNA não metilado na fita filha depois da síntese do DNA de modo que o padrão de metilação parental seja mantido em células-filhas. Também é fundamental na inativação do cromossomo X e *imprinting* genômico nos embriões de mamíferos, esta enzima de grande porte (1.620 aminoácidos) é direcionada a replicação de sitios consistente com uma remetilação rápida de DNA em células somáticas (Wolffe et al., 1999). Em contraste, DNMT3a é mais

ativamente envolvida na metilação do DNA não metilado para estabelecer novos padrões de metilação (Feng *et al.,* 2010).

Okano e colaboradores (1999) determinram que a expressão persistente de DNMT3a e 3b após a gastrulação sugere que a metilação *de novo* não é estritamente limitada aos embriões como se acreditava anteriormente, sugerindo que a metilação *de novo* também desempenha um papel importante durante o desenvolvimento tardio.

Estudos realizados sobre o pontencial dos fibroblastos gengivais na indução de uma resposta biológica demonstraram que os fibroblastos são capazes de fagocitar objetos estranhos e participar da síntese e remodelação das fibras colágenas, desempenhando um papel importante no processo de cicatrização (Wang & Ohura, 2002). Estas células secretam uma variedade de citocinas imunorregulatórias e mediadores químicos (Okada & Murakami, 1998).

Em 2008, Sen e colaboradores demonstraram que dentro do tecido epitelial JMJD3 silenciado bloqueia a diferenciação celular, já JMJD3 expresso induz a diferenciação celular. Estes resultados demonstraram que a expressão ou não do gene JMJD3 atua no controle da diferenciação da epiderme de mamíferos. Em outro estudo sobre cicatrização de feridas concluiu-se que JMJD3 e UTX são regulados na reparação de pele de roedores (Shaw & Martin, 2009). JMJD3 e UTX têm um papel funcional regulando proteínas T-Box indutíveis, aumentando a sua atividade demetilase para promover a transcrição em células diferenciadas (Miller *et al.,* 2010). De acordo com estes estudos, pode ser sugerido que estes genes e sua expressão ou seu silenciamento está presente nas dinâmicas dos queratinócitos e fibroblastos.

De Santa Fe (2009) concluiu em seu estudo que JMJD3 participa da resposta inflamatória induzida pelo LPS na estimulação em macrófagos, fazendo ajustes finos na transcrição de vários genes ao invés de contribuir na sua indução. Além de ter papel importante no desenvolvimento e renovação tecidual.

As demetilases H3K27 (H3 lysine 27) histona específicas são representadas por três proteínas de domínio JMJC e UTX, UTY e JMJD3. UTX

interage com os H3K24 (H3 lysine 24), HMTs (histone methyltansferase) e MLL2-4 (mixed lineage leukemia 2 lysine 4). Em alguns casos, MLL2 foi necessária para o recrutamento do UTX. Portanto, UTX coordena a metilação de H3K24 com a desmetilação de H3K27me3 (Islam *et al.* 2011).

O conhecimento sobre os mecanismos epigenéticos envolvidos em doenças inflamatórias como a periodontite são pouco conhecidos. No presente estudo, foi hipotetizado que LPS derivado de *P. gingivalis*, um dos principais agentes patogênicos em periodontite, poderia modular, em cultura de queratinócitos e fibroblastos gengivais humanos, a expressão gênica de algumas enzimas (DNMT1, DNMT3a, JMJD3, e UXT) que promovem eventos epigenéticos. Além disso, acredita-se que sítios periodontalmente doentes poderiam apresentar uma alteração na expressão gênica destas mesmas enzimas, quando comparados a sítios sadios (DNMT1, DNMT3a, JMJD3, e UTX).

CAPÍTULO 1 - *Porphyromona Gingivalis* LPS stimulation downregulates DNMT1, DNMT3a, and JMJD3 gene expression in human keratinocytes

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Abstract

Objective: The aim of this study was to assess whether *P. gingivalis* LPS can modulate, in culture of the human keratinocytes and human gingival fibroblasts, gene expression levels of the some enzymes that promote epigenetic events. In addition, the same enzymes were evaluated in sample from healthy and periodontitis affected individuals. **Material and Methods**: Primary gingival fibroblast culture and keratinocytes (HaCaT) were treated with medium containing *P. gingivalis* LPS or *P. gingivalis* LPS vehicle for 24hs. After this period, cell viability were assessed by MTT test, and total RNA were extracted to evaluate gene expression levels of the enzymes: DNMT1 (DNA methyltransferase 1), DNMT3a (DNA methyltransferase 3a), histone demethylases JMJD3 and UTX, by qRT-PCR. To evaluate the gene expression in healthy and periodontitis affected individuals, total RNA was extracted from biopsies of gingival tissue from sites with (periodontitis) or without periodontitis (healthy), and gene expression of DNMT1, DNAMT3a, JMJD3 and UTX were evaluated by qRT-PCR. **Results**: No significant differences were found in the gene expression analysis between healthy gingival tissues and gingival tissue from periodontitis sites. 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 found to gingival fibroblasts. **Conclusion**: *P. gingivalis* LPS exposure to keratinocytes, downregulates gene expression of the enzymes that promote epigenetic events.

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 at sites 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 because of 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 [4, 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 [4, 9].

Inflammatory responses in the 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 micro environment observed in the periodontitis exhibits high variation in the transcription levels of molecules implicated in immune response and also periodontal tissue degradation. Recently, some studies have discussed the transcriptional regulations in this microenvironment. Authors have suggested that among several factors the epigenetic could play an important role [11- 14].

Epigenetics is defined as the study of mitotically and meiotically heritable changes in gene function that are not dependent on DNA sequence [15]. Epigenetic mechanisms are not only related with diseases, it are essential for development, cell differentiation, protection against viral genomes, and seem to be critical for the integration of endogenous and environmental signals during the life-time of a cell or an organism [16]. The molecular basis of epigenetic processes is complex and involves modifications of histones, methylation of DNA, positioning of histone variants, and gene regulation by non-coding RNAs.

The DNA methylation transcriptional regulation is carried out by two types of DNA-methyltransferases (DNMTs): *de novo* and maintenance methyltransferases [17, 18]. DNA methylation patterns are established during early development by de novo methyltransferases DNMT3a and DNMT3b [17, 19]. Patterns of DNA methylation are propagated with extreme fidelity by the maintenance methyltransferase DNMT1 [17], which reproduces patterns of methylated and unmethylated CpG sites between cell generations [16]. However, high activity of DNMT3A and DNMT3B genes can be observed in cells when the tissue is exposed to events that modify the micro-environment, altering the metabolism of local cells, like inflammation [20].

One of the epigenetic events involving histones modification is the methylation. Methylation of histone H3 lysines 4 and 27 is catalyzed by the trithorax and polycomb family of developmental regulators [14]. Di- and tri-methylation on H3 lysine 27 (H3K27me2/3) is generally associated with transcriptional repression, whereas trimethylation on lysine 4 (H3K4me3) is associated with transcriptional activation [14, 21, 22]. The histone H3 lysine 27 (H3K27) demethylases JMJD3 (Jumonji Domain Containing 3) and UTX (Ubiquitously Transcribed Tetratricopeptide Repeat, X chromosome) remove the gene-inactivating H3K27 dimethyl and trimethyl marks and are involved in inducing and/or maintaining gene expression [23].

Although there are many studies investigating epigenetic mechanisms in diseases like cancer [15, 24], the involvement of these mechanisms in inflammatory diseases such as periodontitis is poorly known. 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 UXT, in culture of the human 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 UXT).

Material and Methods

Materials

The Dulbecco's modified Eagle medium (DMEM) and Hank's salt solution were obtained from Cultilab (São Paulo, Brazil). The heat-inactivated fetal bovine serum (FBS) and Trypsin/EDTA solution used in all experiments were from LGC Biotecnologia (São Paulo, Brazil). The antibiotics (penicillin/streptomycin/amphotericin B) were purchased from GIBCO (Auckland, New York, USA). The tissue culture multi-wells plates were obtained from TPP® (Switzerland). The RNAlater used to stored gingival samples were purchased from Ambion Inc. (Austin, TX, USA). The *Porphyromona gingivalis* lipopolysaccharide (*P.gingivalis* LPS) were obtained from InvivoGen (San Diego, CA, USA). To viability analysis was used a MTT assay (CellTiter 96® Non-Radioactive Cell Proliferation Assay) purchased from Promega (Madison, WI, USA). The TRIzol® reagent, DNase and SuperScript III First-strand Synthesis of the Oligo (dT) primer used in the qRT-PCR were obtained from Invitrogen (Carlsbad, CA, USA). The Fast SYBR Green Master Mix used in the qRT-PCR reactions was from Applied Biosystems (Frederick, MD, USA).

Study Population and sample collection

To assess whether periodontitis could modulate the expression of the target genes of this study, 20 patients: 11 women (7 healthy and 4 with periodontitis) and 9 men (3 healthy and 6 with periodontitis), aged 21 to 66 years (mean age, 45.1 ± 11.8 years) were selected. All patients from this study had 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 the periodontitis condition. All patients were non-smoking and female patients were not pregnant, lactating, or using any method of birth control. Gingival tissue was obtained from subjects undergoing periodontal surgery for disease and non-disease-related reasons (e.g., esthetics). In this study samples were obtained from healthy and periodontitis affected individuals. Control group (healthy) had systemically and periodontally healthy subjects. These subjects had a high standard of oral hygiene with no bleeding on probing and probing depth ≤ 3 mm in the selected sites (n=10). The periodontitis group included subjects who were systemically healthy and clinically diagnosed with moderate to severe chronic periodontitis, e.g., probing depth ≥5 mm and bleeding on probing (n=10). The gingival biopsies were obtained, rinsed with cold sterile saline solution, and stored in a tube containing RNAlater at -70° C to gene expression analysis.

Primary cell culture

Healthy human gingival tissue biopsies were obtained from subjects undergoing periodontal surgery for disease and non-disease-related reasons (e.g., esthetics). The donor patients had the same characteristics as described to individuals of the control group in the topic: *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 ug/mL, and then the samples were incubated in DMEM containing 10% FBS and penicillin/streptomycin/amphotericin B up to 4h 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 an atmosphere of high humidity and 5% CO_2. When cells growing out from explants reached confluence, the pieces of gingival tissue were removed, the cells (gingival fibroblasts) were trypsinized using a solution of 0.05% trypsin / 0.02% ethylenediamine-tetraacetic acid (EDTA) in phosphate buffered saline solution (PBS) and placed in a second culture dish. The experiment was performed using cells between the third and the sixth passages.

LPS Treatment

Human keratinocytes (HaCaT), kindly provide by Professor Dr. Ricardo Coletta from Department of Oral Diagnosis of the Piracicaba Dental School, São Paulo, Brazil, and Human gingival fibroblast (GF) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin (100 units/mL) / streptomycin (100 µg/mL) at 37°C in an atmosphere of high humidity and 5% CO_2. At harvest, the cells were plated at a concentration of 2 x 10^5/mL in multi-well plates and cultured until they had reached a confluent state (~48hs to HaCaT cells and 72hs to GF). The confluent cells were exposed to 500ng/mL of the *P. gingivalis* LPS diluted in sterile water, for 24 hours at 37°C in an atmosphere of high humidity and 5% CO_2, and then the cell viability and gene expression were evaluated. During the experimental period, the cells were cultured

with DMEM supplemented with 2% FBS. Cultures treated with LPS vehicle (water used to diluted *P.gingivalis* LPS) served as controls.

Viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction to formazan crystals by mitochondrial enzymes reductases using a MTT biochemical assay in 96-well plates (n=5). Initially, it was added at the experimental medium 15 µL of MTT solution and incubated for 4 hours at 37°C in 5% CO_2. Control wells without cells containing experimental medium were incubated in parallel with test samples to measure the absorbance background. Afterwards, it was added 100 µL of solubilization/stop solution to solubilize the formazan product incubating for 1 hour 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 with periodontitis individuals, and 2) HaCaT cells and human gingival fibroblasts treated with *P. gingivalis* LPS (or vehicle). After sample collection (biopsies) or cells treatment (HaCaT and GF) as previously described, total RNA was extracted using the TRIzol® reagent, following the manufacturer's recommendation. The RNA quantification and purity were measured by photometric measurement using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the RNA

quality was assessed by electrophoresis on a denaturing 2% agarosis gel. One microgram of total highly purified RNA was treated with DNase and 500 ng 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 Step One Plus (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using the Jump Start SYBR Green Taq Ready Mix™. 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'-CAGCGTCACACAGAAGCATATCC-3', R: 5'-GGTCCTCACTTTGCTGAACTTGG-3'; JMJD3 F: 5'-AGCTGGCCCTGGAACGATA-3', R: 5'-GGCCCTGGTAAGCGATTT-3'; UTX F:5'-TACAAATCCGAACAACCC-3', R: 5'-TGAGGAGGCCTGGTACTGT-3'; GAPDH F: 5'-GAAGGTGAAGGTCGGAGTC-3', R: 5'-GAAGATGGTGATGGGATTTC-3'. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected, denoted Cp (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 of all tests 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 500ng/mL of the LPS did not affect viability of both cell types (Fig. 1).

LPS from *P.gingivalis* was administered to keratinocytes (HaCaT) and gingival fibroblasts (GF), in order to evaluate if that stimulation could modulate DNMT1, DNMT3a, JMJD3, and UTX gene expression. The exposure of HaCaT cells to LPS for 24hs lead to a decrease of the 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 exposure to LPS vehicle (deionized water). The UTX gene expression in HaCaT cells was not modulating by LPS stimulation ($p>0.05$). Gingival fibroblasts were exposed to LPS or LPS vehicle for 24hs, and the gene expression analysis demonstrated LPS stimulation did not change expression levels of mRNA to 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 [25]. In this way, studies with rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus and periodontitis have reported an epigenetic regulation of the

genes related to cell proliferation, inflammatory responses and tissue remodeling [12, 25-27]. 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 [28]. LPS is an essential macromolecule that comprises the outer surface of gram-negative bacteria, and when this molecule is recognized by the human 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 the cells from the gingival tissue has been shown to depend of the binding of this molecule to toll like receptor 2 (TLR2) and TLR4 [29-31].

Although have been reported that human gingival fibroblast responses to *P. gngivalis* LPS stimulation [32], the results from our study showed that only keratinocytes (HaCaT) responses to *P. gingivalis* LPS in the evaluated parameters. Epithelial cells, which express TLRs, may function as non-professional inflammatory cells and help professional cells of the innate and adaptive immune system to clear the bacterial infection. The activation of the TLRs from oral epithelial cells promotes a release of pro-inflammatory and chemotactic cytokines, matrix-degrading enzymes and prostaglandins [6, 31, 33-35]. Therefore, oral epithelial cells can actively participate in periodontal inflammation.

Upon TLR engagement, LPS triggers an intracellular signaling cascade which involves, among others factors, the nuclear transactivation of NF-κB (Nuclear factor-kappa B) [29, 31]. NF-κB is a family of transcription factors required for the induction of the most important classes of inflammatory genes, included genes involved with 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 have direct regulation 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 and inflammatory cytokines [37, 38]. In addition, JMJD3 is involved in gene silencing during Inflammation [37]. Because Jmjd3 binds to target genes and regulates their H3K27me3 levels and transcriptional activity, the decrease of the gene expression levels of JMJD3 found in our study, could lead to an alteration of the transcription levels of the inflammatory genes activated by LPS.

It has been generally accepted that an increased methylation (hypermethylation) in the gene promoter region is associated with a decrease of gene expression, while hypomethylation pattern is closely associated with transcriptional upregulation [12, 39]. The change of methylation status in CpG islands of DNA, which are regions of genome that contain a high percentage of CpG dinucleotides, are profoundly associated with diseases such as developmental abnormalities, cancer and chronic inflammatory states [12, 17, 40].

We found that the gene expression of the DNMT3a and DNMT1, enzymes that promotes and maintain DNA methylation, was downregulated when keratinocytes were exposed to *P. gingivalis* LPS. Our results corroborated with the findings from Yin L and Chung, 2011, which showed that *P. gingivalis* (whole bacteria) can cause a decrease of the 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 periodontitis pathogenesis.

Our results did not evidence changes in the gene expression of the enzymes evaluated in samples from biopsies (healthy x periodontitis). The different cells types found in tissue from sites affect by periodontitis, such as epithelial cells, fibroblasts, inflammatory cells, mayb have a specific epigenetic profile, what make

it difficult to check the real role of each cell in the epigenetic events in that microenvironment.

In summary, this study demonstrated that LPS derivate from *P. gingivalis* may modulate the gene expression of the important enzymes involved in epigenetic control in keratinocytes. Further investigations should be done, in attempt to understand the role of the epigenetic regulation in periodontitis pathogenesis. The advance of knowledge in this area may allow epigenetic therapies, especially in complex multi-factorial diseases such as periodontitis.

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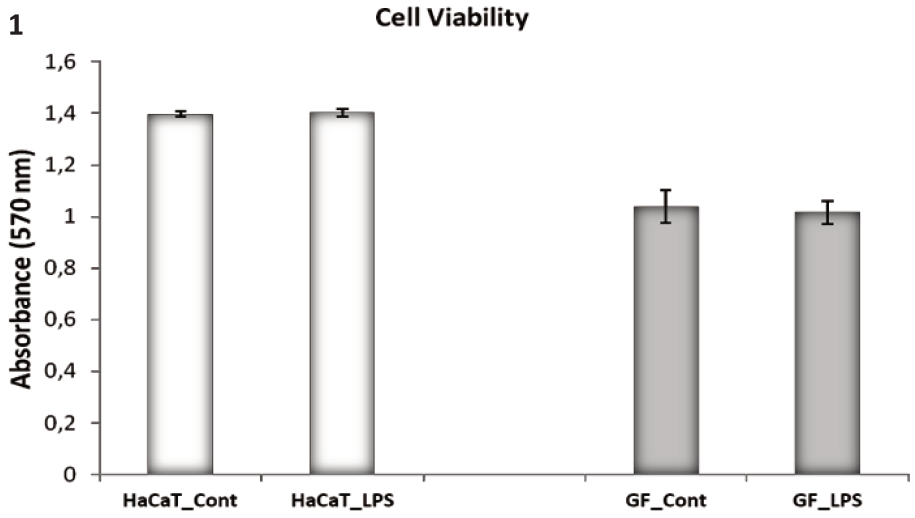

Figure 1 – The graphic represents the means ± SD of the values obtained for cell viability after to exposure keratinocytes (HaCaT) and gingival fibroblasts (GF) cells to 500ng/mL of the *P.g* LPS (HaCaT_LPS and GF_LPS) or vehicle (HaCaT_Cont and GF_Cont) for 24hs. No significant differences on the HaCaT or GF cells viability were found by LPS stimulation. (n=5/group.)

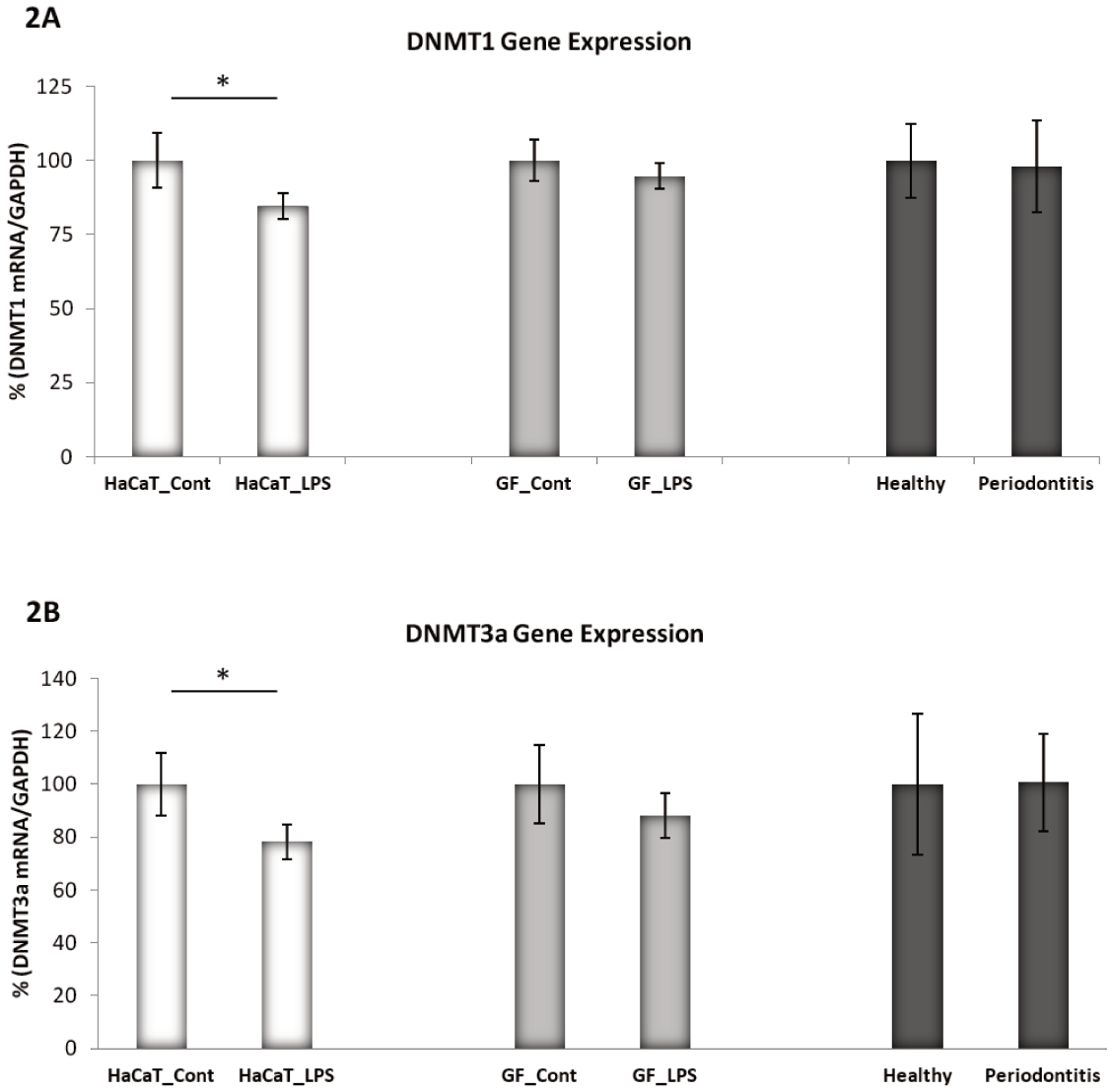

Figure 2 – The graphics represents means ± SD of the values obtained to DNA methytransferase 1 (DNMT1) and DNMT3a gene expression analysis. The results showed that the treatment of the HaCaT cells with 500ng/mL of the *P.g* LPS (HaCaT_LPS) for 24hs reduces theDNMT1 (Fig.2A) and DNMT3a (Fig.2B) gene expression levels when compared to control (HaCaT_Cont, vehicle treatment). LPS treatment to gingival fibroblasts (GF) do not change mRNA transcription levels for DNMT1 (Fig.2A) or DNMT3a (Fig. 2B) when compared to control. Samples from healthy or with periodontitis individuals showed similar gene expression levels to DNMT1 (Fig.2A) and DNMT3a (Fig.2B). For both Fig.2A and Fig. 2B, the data are expressed as of target genes and reference gene (GAPDH) ratio. Gene expression means obtained for vehicle groups was assumed as 100% ±SD in each experimental condition. (*) indicates means statistically different ($p<0.05$). (n=3/groups to HaCaT and GF; n=10/group to healthy and periodontitis groups).

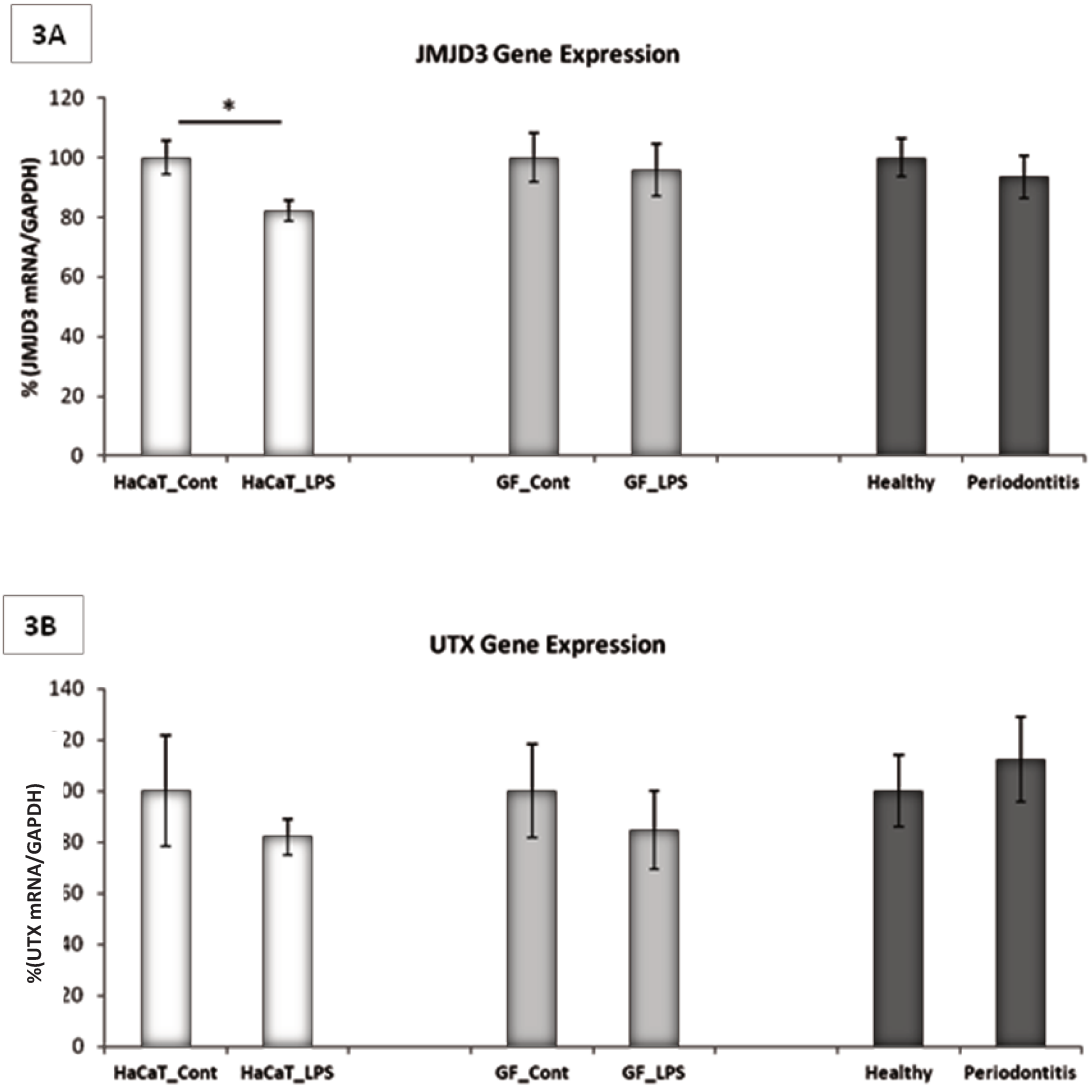

Figure 3 – The graphics represents means ± SD of the values obtained to JMJD3 and UTX gene expression analysis. The results showed that the treatment of the HaCaT cells with of *P.g* LPS 500ng/mL (HaCaT_LPS) for 24hs reduces the JMJD3 (Fig.3A) gene expression levels when compared to control (HaCaT_Cont,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

CONCLUSÃO

Pelos dados obtidos neste trabalho é possível concluir que LPS de *P.gingivalis*, um dos principais agentes periodontopatogênicos, pode modular a expressão de enzimas que promovem eventos epigenéticos em queratinócitos.

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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Estudo do processo de metilação de DNA e expressão de DNA-metiltranferase 1 em pacientes com periodontite, e em fibroblastos gengivais humanos expostos a periodontopatógenos"**, protocolo nº 123/2010, dos pesquisadores Marcelo Rocha Marques e Gláucia de Camargo Pereira, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 15/11/2010.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Study of DNA methylation status and DNA-metiltransferase 1 expression in patientes with periodontitis, and in human gingival fibroblas exposed to periodontopathogens"**, register number 123/2010, of Marcelo Rocha Marques and Gláucia de Camargo Pereira, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 11/15/2010.

Prof. Dr. Pablo Agustin Vargas
Secretário
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Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
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