Comunicação Científica e Técnica em Odontologia 3

Emanuela Carla dos Santos
(Organizadora)

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APRESENTAÇÃO

A ciência da Odontologia é desafiadora e encantadora, para aqueles profissionais que desejam, cada vez, mais aprimorar seu conhecimento. Graças à tecnologia e o acesso facilitado, podemos sempre estar atualizados dentro de nossa área.

A Atena Editora lança mais um livro em formato digital, associando conhecimento e inovação técnica, com artigos contundentes para o crescimento da comunidade odontológica dentro do cenário da pesquisa científica.

Este e-book, Comunicação Científica e Técnica em Odontologia 3, vem complementar os trabalhos já publicados, expandindo áreas do conhecimento abordadas como tecnologia em odontologia, relatos de casos para melhorar soluções clínicas, bem como artigos que concretizam dados e tendências dentro do âmbito odontológico.

Ótima leitura a todos!

Emanuela Carla dos Santos

SUMÁRIO

CAPÍTULO 8

RELATION BETWEEN PERIODONTAL CONDITION AND THE IN VITRO PRODUCTION OF HUMAN HSP60 INDUCED BY RECOMBINANT HMUY OF *Porphyromonas gingivalis*

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ABSTRACT: *Porphyromonas gingivalis* is an important pathogen in chronic periodontitis whose virulence factors, such as HmuY, elicit the host response. The stress caused by the microbial challenge includes the production of chaperones responsible for cellular homeostasis and protein repair: the heat shock protein (HSP). The present study aimed to evaluate the production of HSP60 by cells of individuals with chronic periodontitis (CP) and without periodontitis (WP), under stimulus of HmuY. Eleven individuals with CP and twenty-six WP were examined using the following clinical periodontal parameters: probing depth (PD), bleeding on probing (BP) and clinical attachment level (CAL). Peripheral blood mononuclear cells (PBMC) were collected and cultured for 48 hours with HmuY. The levels of human HSP60 in the supernatant and cytosol were assessed through enzyme linked immunosorbent assay. No statistically significant differences were

observed between the CP and WP individuals when the cells were cultured in the presence of HmuY (p= 0,261). However, a moderate positive correlation (r=0,666; p=0,025) was observed between the percentage of sites with CAL≥5 mm and the HSP60 levels induced by the recombinant protein HmuY in the PBMC. The highest levels of HSP60 were observed among the sites with the highest severity level of clinical attachment loss. The lipoprotein HmuY of *P. gingivalis* plays a role in the stress of the host cell.
KEYWORDS: Periodontitis, HSP60, *Porphyromonas gingivalis*, host response.

1 I INTRODUCTION

Periodontitis is an infectious and inflammatory disease that results in the destruction of the supporting tissues of the teeth (bone, periodontal ligament and cement). This destruction induced in the periodontal tissues is related to biofilm dysbiosis and the host response pattern, which can be influenced by environmental, systemic and genetic factors (Hajishengallis and Lamont 2014; Meyle and Chapple 2015).

The gingival inflammation is a result of the adhesion of microorganisms to the biofilm present in the dental surface, including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia, Prevotella intermedia*, *Prevotella nigrescens*, *Treponema denticola, Campylobacter rectus* and *Eikenella corrodens* (Teles et al. 2012). These oral microbiome microorganisms have an array of virulence factors that increase their infectivity, multiplication capacity and persistence in the periodontium (Sudhakara et al. 2018).

P. gingivalis is a secondary colonizer of the oral cavity, whose presence requires the creation of environmental conditions by pioneer species (Teles et al. 2012; Socransky and Haffajje 2005). This microorganism produces a wide range of virulence factors, such as fimbriae, lipopolysaccharide, gingipains and haemagglutinins, which are involved with bacterial colonization, tissue destruction and modulation of the host immune response (Hajishengallis and Lamont 2014; Sudhakara et al. 2018; Hosogi, Hayawa and Abiko 2001; Gao et al. 2010).

Additionally, beyond these already widely studied virulence factors, HmuY is an iron-binding lipoprotein. This lipoprotein can induce increased levels of IL-10, IL-6, IgG and IgG1 anti-HmuY and can inhibit IL-8 production by cells of the host immune system (Trindade et al. 2012; 2013). HmuY also seems to boost the invasion of macrophages by *P. gingivalis*, which respond to this infection via the TLR-7 pathway (Gmiterek et al. 2016).

In the process of programmed cell death, HmuY induces high levels of Bcl-2, resulting in late apoptosis in peripheral blood mononuclear cells (PBMC), cellular necrosis and maintenance of the inflammatory process, prolonging the process of tissue destruction (Trindade et al. 2012; Carvalho-Filho et al. 2013).

However, several other mechanisms of interaction of *P. gingivalis* with the host, both of innate immunity and adaptive immunity, are still not well understood. It is known that in response to stressors, such as bacterial aggressions, cells have quite effective resistance mechanisms, such as heat shock proteins (HSP) (Indumathy et al. 2014), responsible for maintaining cellular homeostasis (Parsell and Lindquist 1993), whose presence has been demonstrated in tissue samples derived from periodontitis lesions (Lundquist et al. 1994). Furthermore, HSPs may act as intercellular signaling molecules, playing a role in cell cycle progression, apoptosis and are involved in some disease processes (Cappello et al. 2008; Leishman et al. 2017).

HSPs are categorized into distinct families according to their molecular weight: small HSPs (from 15 to 30 kDa), HSP40, HSP60, HSP70, HSP90 and HSP100 (Khalil et al. 2011). HSP60 is a mitochondrial chaperonin, but it can be found in the cytosol. It aids in the folding of mitochondrial proteins and facilitates the proteolytic degradation of malformed or denatured proteins. Depending on its location, it may present pro and anti-apoptotic functions (Chandra et al. 2007). HSP60 is a conserved protein throughout evolution and has homologous between prokaryotic and eukaryotic cells (Gemell et al. 2002). The immune responses to human HSP60 may be related to cross-response to microbial HSP60 since molecular mimicry (cross-reactive epitopes) can mediate inflammatory diseases, including autoimmune diseases. For example, the expression of host protective HSP60 on vascular endothelial cells may act as targets for cross-reactive autoimmune responses (Leishman et al. 2017; Tabeta et al. 2000).

The HSP60 of *P. gingivalis* has been detected by a proteomic approach in an immunogenic chromatographic fraction of *P. gingivalis* ATCC33277 sonicated extract in a parallel study conducted by our group (unpublished data).

Due to its conservation among microbial pathogens and to its ability to induce cellular and humoral immune responses, HSP60 may play a role as a candidate antigen in chronic periodontitis. Therefore, the current study aimed to evaluate the induction of human HSP60 production by *P. gingivalis* HmuY antigen in individuals with chronic periodontitis to better understand the process of cellular stress.

2 I MATERIALS AND METHODS

2.1 Sample

This research was approved by the Feira de Santana State University Institutional Review Board (Number 79791). All participants signed the consent to participate form. Volunteers who received dental care in the undergraduate clinics of the Dental School (State University of Feira de Santana, Bahia, Brazil) participated in this study. The following individuals were excluded from our sample: pregnant women; patients with systemic diseases such as diabetes, cardiovascular diseases or autoimmune

diseases; smokers, alcoholic and drug addicted ones; patients with previous periodontal treatment; patients who previously used anti-inflammatory drugs up to two months prior to clinical evaluation and antibiotics up to six months prior to clinical evaluation.

2.2 Periodontal Disease Classification

The periodontal condition was evaluated by a single trained examiner (ACMP) (kappa = 0.932) using a Williams periodontal probe (Hu Friedy, Chicago, IL, USA). Periodontal examination included the descriptors: probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP).

Chronic periodontitis (CP group) was defined as the presence of at least four teeth with at least one site with a probing depth greater than or equal to 4 mm, clinical attachment level greater than or equal to 3 mm, associated with bleeding (Gomes-Filho et al. 2007). Individuals who did not meet these criteria were not considered to have periodontitis (WP group).

For the assessment of the periodontal condition, in addition to the evaluation of the presence or absence of the disease, the percentage of sites with BOP, sites with PD ≥ 4 mm, sites with CAL ≥ 3 mm, sites with CAL ≥ 4 mm and sites with CAL ≥ 5 mm were registered.

2.3 Antigen

The recombinant HmuY protein (rHmuY) from *P. gingivalis* was obtained by overexpression in *E. coli* and purification as previously described (Olczak et al. 2010). Based on previous assays (data not presented in this paper), the protein was used in the culture of cells with the final concentration of 2.5 µg / mL.

2.4 Culture of Cells

The peripheral blood sample from each participant was applied in a cell separation medium (Ficoll-Paque, Sigma Chemical Co., St. Louis, MO, USA) to obtain peripheral blood mononuclear cells (PBMC) by density gradient according to the manufacturer's guidelines. The PBMC were then washed twice in Roswell Park Memorial Institute (RPMI) medium (LGCBio, São Paulo, SP, Brazil) and cultured in flat bottom plates with 24 wells (10^6 cells / well), in RPMI medium supplemented with 10% fetal bovine serum (heat inactivated complement) and 1% antibiotic / antimycotic solution (R & D Systems, Minneapolis, MN, USA). PBMC were cultured for 48h at 37°C under 5% CO_2 atmosphere in the presence of 5 µg / mL pokeweed mitogen (PWM) (positive control), of 2.5 µg / mL rHmuY, or without stimulus (negative control).

2.5 Quantification of Human HSP60

To determine HSP60 levels produced by PBMC after 48h, lysed cell contents (cells and supernatant) were evaluated by enzyme-linked immunosorbent assay (ELISA)

using the Human total HSP60 Duoset according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA, Statistical analysis). The samples were diluted according to the manufacturer's instructions and were applied to 96-well polystyrene plates with high adsorption capacity (SKC-109A - Anti-HSP60, Immunoassay Plate). Reactions were revealed using 3,3', 5,5' - tetramethylbenzidine and optical density was obtained using an ELISA reader (Elx 800 - Bio-Tek) at 450 nm.

2.6 Statistical Analysis

The Student's T test was used to compare numerical variables between the comparison groups related to age and periodontal clinical findings, while the chi-square test was used to compare the sex variable. Differences in cytokine levels between groups were assessed using the Mann-Whitney U test and correlations between clinical findings and HSP60 levels were tested using Spearman's correlation coefficient. Values of $p \leq 0.05$ were considered statistically significant. Data were analyzed using SPSS (Statistical Package for Social Sciences) version 17.0 for Windows.

3 I RESULTS

Twenty-seven subjects participated in the study, of which eleven comprised the group with chronic periodontitis (CP), corresponding to 40.7%, while the group without periodontitis (WP) was composed of sixteen participants (59.3%). The mean age of participants in the CP group was 39.8 years ± 7.5 years, with a minimum limit of 25 years and a maximum of 48 years. The mean age of participants in WP was 38 years ± 11.5 years, with minimum and maximum limits of 20 years and 57 years, respectively.

Among the participants in the CP group, seven (63.6%) were female and four (36.4%) were male. In the WP group, nine (56.3%) were female and seven (43.7%) were male. There was no statistically significant difference in the mean age ($p = 0.65$) and the proportion of males or females ($p = 0.70$) between the two groups, demonstrating that both were homogeneous with respect to these two covariables (Table 1).

	GROUP WP	GROUP CP	p*
	n = 16	n = 11	
Age (years) (mean ± SD)	38±11.5	39.8±7.5	0.65
Sex (female, male)	9/7	7/4	0.70
% sites with BOP (mean ± SD)	8.6±11.1	29.9 ±16.3	< 0.01
% sites with PD ≥ 4 mm	1.27±1.5	14.16±9.1	<0.01

(mean ± SD)			
% sites with CAL ≥ 3 mm	20.3±14.3	57.1±20.6	<0.01
(mean ± SD)			
% sites with CAL ≥ 5 mm	1.6±2.3	9.2±9.4	0.02
(mean ± SD)			

Table 1. Characteristics of the participants of the groups without periodontitis (WP) and with chronic periodontitis (CP). Brazil, 2019

*P-value: level of significance ≤ 0.05. Chi-square test was used to compare the sex variable and *Student's T-test* was used for other values. Bleeding on probing (BOP), probing depth (PD) and *clinical attachment level* (CAL).

Regarding aspects related to the periodontal condition, there was a statistically significant difference between the two groups in all the clinical descriptors evaluated. Table 1 also shows that the CP group presented a mean percentage of sites with bleeding on probing of 29.9, while the value for the WP group was 8.6 (p <0.01); the mean percentage of sites with probing depths greater than or equal to 4 mm was significantly higher (p <0.01) in the CP group (14.16%) when compared to the WP group (1.27%). Considering the level of clinical attachment, the CP group presented mean percentages of CAL greater than or equal to 3 mm (p <0.01) and CAL greater than or equal to 5 mm (p = 0.02) of 57.1 and 9.2, respectively, while the percentages for the WP group were 20.3 and 1.6, respectively.

Differences in the levels of HSP60 measured in PBMC cultures were analyzed according to each stimulus used. Statistically significant differences were detected between the CP and WP groups when the cells were cultured without stimulus (p = 0.09), in presence of pokeweed mitogen (p = 0.76) or rHmuY protein (p = 0.26) (Figure 1).

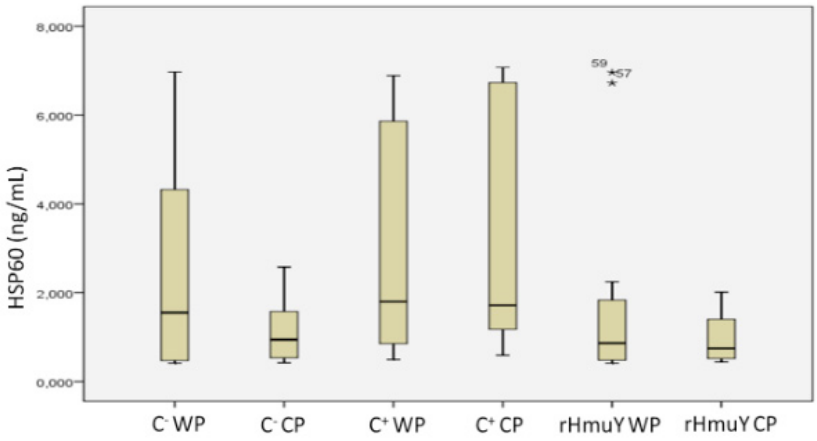

Figure 1. HSP60 levels in culture supernatants and peripheral blood mononuclear cell (PBMC) lysate of volunteers without periodontitis (WP) and with chronic periodontitis (CP) exposed to different conditions: without antigen (C^-), stimulated with pokeweed mitogen (C^+) and stimulated with rHmuY (Brazil, 2019)

However, the cells of volunteers without periodontitis, when cultured only with the culture medium without stimulation, presented higher levels of HSP60 when compared to the cells of individuals diagnosed with periodontitis cultured under the same conditions. In the presence of rHmuY, there was a decline in the levels of HSP60 produced by the cells of individuals in the WP group. This decline was not as marked when the cells of the CP group were examined.

When analyzing the correlation between HSP60 levels produced by the PBMC only from patients with diagnosis of periodontitis and their periodontal condition (percentage of sites with bleeding on probing, percentage of sites with probing depth ≥ 4 mm, percentage of sites with CAL ≥ 3 mm, percentage of sites with CAL ≥ 4 mm, percentage of sites with CAL ≥ 5 mm), it was possible to observe a moderate positive correlation ($r = 0.67$, $p = 0.03$) between the percentage of sites with CAL ≥ 5 mm and the HSP60 levels produced by the PBMC stimulated with the recombinant protein HmuY of *P. gingivalis*, as shown in figure 2.

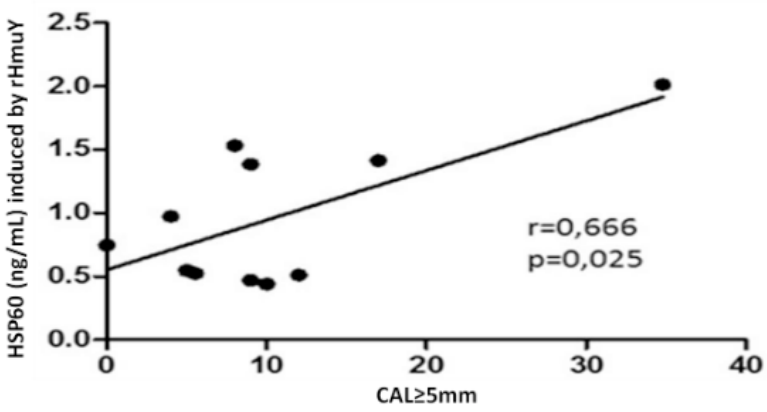

Figure 2. Correlation between the levels of HSP60 induced by HmuY and the percentage of clinical attachment level greater than or equal to 5 mm (Brazil, 2019)

4 I DISCUSSION

The preliminary findings of the present study points to a paradoxical role of HSP60 thermal shock protein in the pathogenesis of periodontitis. In contrast, the cells of individuals without periodontitis and without contact with antigens in culture, simulating a primary immune response, presented high levels of the protein, suggesting a cytoprotective role (Parsell and Lindquist 1993). A tendency in the decrease of the production levels of HSP60 in individuals with periodontitis when the cells were exposed to the HmuY, when compared with the individuals without the disease, was also detected. These findings contrast with those of other studies showing increased production of this protein in cells exposed to stressful conditions such as infection (Henderson et al. 2015).

On the other hand, even though there is a tendency of individuals with periodontitis present decreased levels of this protein when compared to individuals

without periodontitis and without stimulus, our results show that the level of this protein increases according to the severity of periodontitis, as determined by clinical attachment level.

Previous studies evaluated the role of human HSP60 in the pathogenesis of periodontitis (Leishman et al. 2017; Henderson et al. 2015). The present study unveils the correlation between the production of this protein induced by rHmuY and the periodontal condition.

Other studies using different research models have shown that the chaperone function of HSP60 is necessary when host cells are stressed by contact with the pathogen. In an infectious process, pathogens release toxins that trigger the inflammatory process, as soon as the host responds to these toxins, stimulating tumor necrosis factor (TNF), interferon gamma (INF-γ), among other pro-inflammatory mediators. Intracellular heat shock proteins have a cytoprotective action, such as HSP70 that was able to protect macrophages infected by *Salmonella choleraesuis* of TNF induced cell death (Kimura et al. 1998).

However, the HmuY protein has been shown to be important in the pathogenesis of periodontitis, acting on the immune response inducing the production of IL-10 and IL-1β and inhibiting the production of IL-8 (Trindade et al. 2012), but its role as a virulence factor still needs to be better understood.

In regular conditions, the gingipains proteases act in the degradation of host heme proteins for iron acquisition by *P. gingivalis* (Lewis et al. 1999; Sroka et al. 2001). HmuY is a heme binding protein whose action is necessary in unfavorable microenvironments where the concentration of iron is low so *P. gingivalis* constituent receptors cannot obtain it (Wójtowicz et al. 2009; Olczak et al. 2010). This fact might be the reason why it was not possible to distinguish healthy individuals from those patients with periodontal disease based on the levels of HSP60. Because HmuY is not a constitutive protein of the bacterium, such as HSP60, it was not possible to assure that the presence of HmuY during natural infection in individuals with chronic periodontitis was high enough to determine a difference in host response in the secondary infection simulated by culture. Thus, this *P. gingivalis* antigen was only able to induce the production of HSP60 at substantial levels in clinically more severe cases.

One of the limitations of the current study is the fact that molecular interactions occur preferentially in a paracrine manner (Gemmell et al. 2002), in an attempt to restrain the infection to the periodontal environment. The good level of systemic health of individuals with periodontitis, despite the chronic source of infection, supports this assumption. Thus, the effects of this infection may not have been pronounced on peripheral blood cells. Other approaches using gingival or periodontal ligament cells would be necessary to elucidate these interactions.

The sample size may also be a limitation, decreasing the power of the study, as well as the self-reported health history, which may have incurred a memory bias.

Investigation of a larger sample is needed to improve the power of the study and to clearly elucidate a role of human HSP60 in the pathogenesis of the periodontal disease.

However, the findings of the present study have clinical relevance, since heat shock proteins, particularly human HSP60, have been identified as therapeutic targets and may have anti-apoptotic function related to their mitochondrial location (Chandra et al. 2007; Sarangi et al. 2013).

The accumulation of heat shock proteins, either physiologically or by therapeutic approaches, may protect the organism from various systemic conditions or diseases, such as myocardial infarction, stroke, sepsis, viral infections, trauma, neurodegenerative diseases, arthritis and diabetes (Tytell and Hooper 2001).

Since *P. gingivalis* is a keystone-pathogen in the beginning and progression of periodontitis, the present investigation may be regarded as a pioneer study as it seeks to elucidate a role of a relevant protein of *P. gingivalis* in the induction of human HSP60. This may be an early indication of the participation of this autologous protein in protecting the host against the stress caused by bacterial infection in periodontitis.

In conclusion, human HSP60 seems to have a cytoprotective role against the stress caused by the bacterial challenge of *P. gingivalis* in the pathogenesis of periodontitis and its production seems to increase with the severity of the disease.

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6 I AUTHORS' CONTRIBUTIONS

SCT designed the study. ACMP, TPR, MSM and PCCF carried out sample collection and diagnostics. TO coordinated the overexpression and purification of rHmuY. PCCF, ACMP, PMM carried out the culture experiments and the immunoassays. SCT performed the statistical analysis. ISGF, RMN, MTX and SCT coordinated the work. ACMP, TPR and EKNSL wrote de manuscript. MTX, SCT, YPB and TO critically revised the manuscript. All authors read and approved the final manuscript. The authors declare that they have no competing interests.

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