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CLINICAL RESEARCH:

Influence of Periodontitis on Th17/Treg Percentages in Patients with Rheumatoid Arthritis

Influencia de la periodontitis sobre los porcentajes de células Th17/Treg de pacientes con artritis reumatoide

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ABSTRACT: Th17/Treg cells and *P. gingivalis* have been implicated in rheumatoid arthritis (RA) and periodontitis (PE). The aim was to determine and compare Th17/Treg cells and the influence of *P. gingivalis* on this cell population in healthy subjects (HS) and patients with PE, RA, and PE/RA. The number of copies of *P. gingivalis* was performed by real-time PCR and flow cytometry was used to analyze Th17/Treg cells. In this study, PE in patients with RA increased the number of DNA copies of *P. gingivalis*, increasing the percentages of Th17 cells, as well as the number and regulation of Treg cells. Periodontitis influences the immune system, increasing the percentage of pro-inflammatory cells in patients with rheumatoid arthritis, which may could influence the inflammatory condition of this population.

KEYWORDS: Periodontitis; Th17 cells; Treg cells; Rheumatoid arthritis.

RESUMEN: Las células Th17/Treg y *P. gingivalis* han sido implicadas en la iniciación y progresión de la artritis reumatoide (AR) y la periodontitis (PE). El objetivo del presente estudio fue determinar y comparar el porcentaje de células Th17/Treg y la influencia de *P. gingivalis* sobre esta población celular en sujetos sanos (HS) y pacientes con EP, AR y PE/AR. El número de copias de *P. gingivalis* se realizó mediante PCR en tiempo real y se utilizó citometría de flujo para analizar las células Th17/Treg. En este estudio, la PE en pacientes con AR aumentó el número de copias de ADN de *P. gingivalis*, aumentando los porcentajes de células Th17, así como el número y la regulación de las células Treg. La periodontitis influye en el sistema inmunológico, aumentando el porcentaje de células proinflamatorias en pacientes con artritis reumatoide, lo que tal vez podría influir en el estado inflamatorio de esta población.

PALABRAS CLAVE: Periodontitis; Células Th17; Células Treg; Artritis reumatoide.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease of multifactorial origin (1). Recent studies have described that the induction and progression of RA are characterized by an abnormal regulatory T (Treg) cell response with a shift toward Th17 cell responses (2). Th 17 cells and Treg cells have specific functions and gene expression. They develop from the same naïve CD4+ T cells but under a different cytokine environment (3). Typical proinflammatory Th17 cells, through the induction of pro-inflammatory cytokines, lead to autoimmune-derived tissue inflammation and joint damage. Furthermore, the activity of Th17 cells and other effector T cells is suppressed by Treg cells (4). Studies have suggested that Th17/Treg cells act as biomarkers in RA, modulating the expression of interleukin IL-17 (5). The CD4+ Th17 cell subset has been implicated in the pathogenesis of multiple diseases such as periodontitis (PE). PE is a chronic infectious inflammatory disease that affects the periodontium and gradually destroys the tooth-supporting alveolar bone. PE is caused by bacterially derived factors and antigens that stimulate an inflammatory reaction activating the immune system (6). High concentrations of IL-17 are found in the crevicular fluid of periodontal pockets from patients with PE (7). Th17 cells characterized as IL-17- producing T-cell subset have been recently identified in chronic periodontal disease lesions (8). These findings indicate that IL-17 may play a dual role: improving pathogen control and promoting alveolar resorption (9). *P. gingivalis* is a bacterium associated with the initiation and progression of PE that increases the periodontal bone destruction and has been implicated in RA onset modulating the host immune response and producing citrullinated antigens that are nearly exclusive to RA (10). The percentage of Th17/Treg cells and the influence of this bacterium in this cell population of PE/RA patients is still unknown. Thus, in this study, we aimed to determine and compare the percentage of Th17/Treg cells and the influence of *P. gingivalis* in healthy subjects (HS) and patients with PE, RA, and PE/RA.

MATERIALS AND METHODS

SUBJECT POPULATION AND CLINICAL EVALUATION

A cross-sectional and observational study was conducted. Patients were selected from the Rheumatology and Osteoporosis Regional Unit at the Central Hospital Ignacio Morones Prieto and Oral Medicine Clinic of the Autonomous University of San Luis Potosí in San Luis Potosí, México. Statistical analysis indicated that 20 patients were adequate in each group for two-sided alpha 0.05

and 95% power (PS-Power and sample size calculation version 3.09 to show a significant difference. Overall, study participants were divided into four groups: HS (n=20), PE patients (n=20), RA patients (n=20), and PE/RA (n=20). RA diagnosis was performed by a rheumatologist under the criteria of the American College of Rheumatology and European League Against Rheumatism established in 2010 (11). All participants were selected from a homogeneous population with similar ages and genders, and PE diagnosis was performed by a calibrated examiner. The inter-examiner consistency was 0.85. Clinical periodontal parameters were examined in all patients: probing depth (PD) and clinical attachment loss (CAL) indexes were assessed using a North Carolina periodontal probe (Hu Friedy, Chicago, IL, USA) graduated in millimeters (0-15mm). The probe was inserted parallel to the teeth long axis and crossed each tooth´s surface circumferentially. CAL index was measured from the epithelial attachment to the cement-enamel junction. Generalized PE diagnosis was determined based on the amount of CAL and is designated as moderate (3 or 4mm) in more than 30% of the teeth involved (12). Patients who had received previous periodontal treatment and antibiotic therapy within the last three months were excluded; the subjects that were included signed a written informed consent. The study was approved by the Research Committee of the Master´s Degree Program in Advanced General Dentistry at San Luis Potosí University, México.

SUBGINGIVAL PLAQUE SAMPLE

After cleaning all teeth crowns with a sterile sponge, subgingival dental plaque (SDP) was taken with a Gracey curette and placed into km an Eppendorf tube with 1ml of Phosphate Buffer Saline (PBS). Samples were stored at -80ºC until the DNA extraction. All samples were processed aseptically to prevent contamination from the environment during the DNA extraction and qualitative/quantitative polymerase chain reaction (PCR) assays.

DEOXYRIBONUCLEIC ACID (DNA) EXTRACTION AND QUALITATIVE/QUANTITATIVE PCR ASSAYS

Plaque samples were washed with PBS. DNA was extracted by phenol-chloroform purification and isopropanol precipitation method as previously reported (13). DNA was rehydrated by Tris- EDTA and the concentration obtained was determined by spectrophotometry (Nanodrop 2000; Thermo Scientific, Madison WI, USA). The presence and count copy number of *P. gingivalis* were detected by PCR and Real-PCR, respectively, using a specific sequence and previously described protocols (14,15). DNA amplification was performed with a thermal cycler (iCycler; BIO-RAD Laboratories, Hercules, CA, USA) with cycling protocols specific for each set of primers (16). Positive and negative controls were included in each PCR set. The PCR products were analyzed by electrophoresis in a 2% agarose gel-Tris-acetate EDTA buffer, and a 100bp DNA ladder marker (New England Biolabs, Beverly, MA, USA) was used as a reference for molecular size (17). The gel was stained with 0.5∫g of ethidium bromide/ml and observed under UV light (E-Gel Imager System with UV base; Thermo Fisher Scientific, Life Technologies, Waltham, MA, USA).

For q-PCR, 20 ∫L of a mixture containing 50 ng of genomic DNA, 1x TaqMan » Universal PCR Master Mix (Applied Biosystems, Foster City, USA), and 0.5mL of Custom TaqMan Gene Expression assay for *P. gingivalis* (Applied Biosystems) was placed in each well of a 48 well plate. The amplification and detection were performed using the StepOne System (Applied Biosystems) with the cycling profiles indicated in the manufacturer´s instructions. Standard curves were prepared using plasmid DNA cloned into a vector in *Escherichia Coli* competent cells by

a 10-fold dilution series. The q-PCR results were expressed as the number of copies of bacteria DNA per milligram of dental plaque.

FLOW CYTOMETRIC ANALYSIS OF TH17 AND TREG CELLS

For the analysis of Th17 and Treg Cells, PBMCs were suspended at a final density of 1x106 cells/mL in a complete culture medium (RPMI supplemented with 10% fetal calf serum and 1% glutamine/penicillin/streptomycin. Cell cultures were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA, 50ng/mL; Sigma, St. Louis, MO) and Ionomycin 1g/mL for 1 million cells in the presence of BD Golgi stop (Protein Transport Inhibitor) at 37C, 5% CO2.

Th17 and Treg cells were washed and stained with a human Th17/Treg phenotyping kit according to the protocol provided by the manufacturers (BD Pharmingen, San Diego, CA). Briefly, cells were fixed, permeabilized, and stained with a cocktail of fluorescent antibodies. The cocktail was composed of PerCP-Cy 5.5 conjugated anti-human CD4, phycoerythrin (PE) conjugated anti-human IL17A, and Alexa Fluor 647 conjugated anti-human FoxP3.

CD4+ T cells producing IL-17A were classified as Th17 cells, whereas CD4+FoxP3+ cells were classified as Treg cells. Stained cells were analyzed in a FACS Canto II (BD Biosciences.

STATISTICAL ANALYSIS

Data were analyzed with the GraphPad Prism version 6.0 software. Qualitative data are expressed as frequency and proportion; quantitative data are expressed as mean, standard deviation, and range. For the determination of data distribution, a Kolmogorov-Smirnov test was applied. To detect statistical differences among groups for quantitative variables, a Kruskal-Wallis was also performed. Flow cytometry data were evaluated by using the Mann-Whitney U-test. For gender, Fisher´s test was applied. $P<0.05$ was considered statistically significant.

RESULTS

The mean age of the study population was approximately 50 years, and the gender distribution was predominantly female (75%). There were no statistical differences in age and sex, making the four groups homogeneous (Table 1). The periodontal status and copies of *P. gingivalis* are reported in Table 2. As expected, similar means of PD measurements are exhibited in HS and RA groups (2.2±0.42 *vs*. 2.42±0.49, $p>0.05$) from the full mouth. Clinical attachment loss (CAL) (1.29±0.36 *vs*. 1.86±0.52, $p>0.05$) was also obtained. On the other hand, PE/RA and PE groups showed different measurements of PD (3.6±1.17 *vs*. 3.2±0.68, $p<0.05$) and CAL (3.12±0.58 *vs*. 2.54±0.46, $p<0.05$). When comparing groups, PE/RA presented a higher number of copies (5.3x107±5.1x108), and there are statistical differences among groups ($p=0.005$). Percentages of Th17 in peripheral blood mononuclear cells (PBMCs) were significantly higher in patients with PE/RA compared to HS, PE, and RA groups (Th7: 5.16±0.77% *vs*. 1.94±0.38% or 2.81±0.11 or 2.88±0.53%), differences were found among groups $p<0.05$. PE and RA groups have similar percentages of these cells, and there were no differences $p>0.05$ (Figure 1). The percentage of Treg cells was similar in all groups (HS: 1.68±0.33%, PE: 2.22 ±1.10%, RA: 1.56±0.32%, and PE/RA: 1.97±0.72%), and there was no difference among groups $p>0.05$. All RA patients included in this study were receiving treatment with disease-modifying anti-rheumatic drugs (DMARDs) receiving mainly prednisone (5.6±1.5mg/day), methotrexate (14.5±5mg/week), and sulphasalazine (2.3±0.5g/day).

Table 1. Clinical characteristics of the study groups.

		HS n=20	PE n=20	RA n=20	RA/PE n=20	*p*
			Frequency (%)			
Sex	Female	16 (80)	15 (75)	14 (70)	15 (75)	0.4524+
	Male	4 (20)	5 (25)	6 (30)	5 (25)	
			X ± SD (range)			
Age**		50.54±11.33	51.04±12.53	49.75±11.94	51.96±11.67	0.6742*
		(30-74)	(30-80)	(21-81)	(21-88)	

HS: Healthy subjects; PE: Periodontitis; RA: Rheumatoid Arthritis; ** Expressed in years.
+Fisher test p >0.05; *Kruskal-Wallis test p <0.05.

Table 2. Periodontal status among groups.

	HS n=20	PE n=20	RA n=20	RA/PE n=20	*p*
		X ± SD (range)			
PD	2.2±0.42	3.6±1.17	2.42±0.49	3.2±0.68	<0.0001*
	(1.2-3.2)	(1.78-5.91)	(1.42-3.5)	(1.9-5.7)	
CAL	1.29±0.36	3.12±0.58	1.86±0.52	2.54±0.46	<0.0001*
	(0.55-2.98)	(1.99-4.10)	(0.74-3.51)	(1.32-3.71)	
*P.gingivalis*º	4.7x104± 1.1x105	8x104±1.7x105	5x104±1.1x105	5.3x107±5.1x108	0.005*
	(0-2.8x105)	(0-4.0x105)	(0-2.5x105)	(1-6.2x108)	

HS: Healthy subjects; PE: Periodontitis; RA: Rheumatoid Arthritis.
PD: pocket depth; CAL: Clinical attachment loss; º: expressed as the number of copies of bateria DNA.
per milligram of dental plaque; *Kruskal-Wallis test p <0.05.

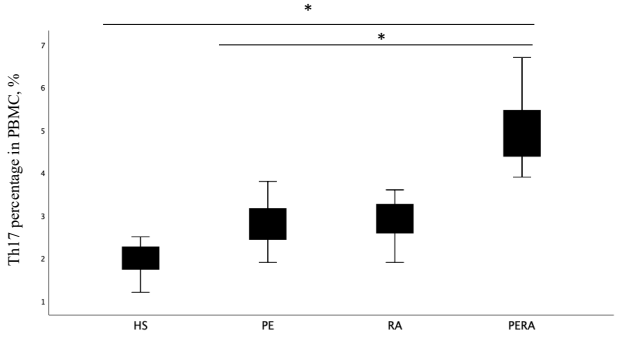

Figure 1. Percentage in PBMCs by study group (HS: Healthy subjects; PE: Periodontitis; RA: Rheumatoid Arthritis) measured by flow cytometry. Results were expressed as mean ± SD (Standard Deviation). Inter-group comparisons were analyzed by one-way ANOVA followed by Tukey`s post hoc test. P<0.05 was considered as statistically significant.

DISCUSSION

Studies have shown the relationship between PE and RA, where *P. gingivalis* plays an important role (18). This study assesses the influence of *P. gingivalis* in the Th17/Treg cell population, considered as a biomarker in RA. In this study, all subjects were selected from a homogeneous population with similar age and gender, applying strict criteria to define PE (12). The mean age was 50 years and predominantly female (75%). These results are like other reports suggesting that people over 40 years are more frequently diagnosed with PE and RA, and women are three times more likely to develop RA than men (19-22). Although there is a postulation that genetic and environmental factors favor the development of both diseases, further studies are needed to prove this hypothesis. In this study, PE/RA patients had higher results in PD and CAL. These are similar to other studies suggesting that PE is more aggressive when patients suffer other medical conditions such as RA (23-25).

When comparing groups PE/RA presented the higher number of copies of *P. gingivalis*. There are no previous studies to compare our results. However, literature has suggested that patients with PE/RA carry more *P. gingivalis*, which is recognized as a major pathogenic organism in PE and has been associated with RA because it is the only bacteria known to express a peptidylarginine deiminase (PAD) (26). The latter could explain why the PE/RA group shows a higher number of copies of *P. gingivalis*. The capability of *P. gingivalis* to express PAD suggests that its infection could impact RA onset and its progression by facilitating autoantigen presentation and the expression of disease-specific autoantibody targeting citrullinated peptides. These antibody responses are almost exclusive to RA patients and could affect RA activity (27). However, it was not evaluated in the present study. Th17 cell has been attracting attention from researchers describing its influence in autoimmune diseases, like RA, as well as its presence in peripheral blood and local lesions of PE patients (28,29). Several studies have shown that Treg cells are involved in periodontal inflammation and tissue destruction during PE (30). In RA, a decrease in Treg and an increase in Th17 cells drive the expansion of autoimmunity in these patients (4). This is the first study evaluating the percentages of Th17/Treg cells in patients with PE/RA. Percentages of Th17 in peripheral blood mononuclear cells (PBMCs) were significantly higher in patients with PE/RA compared to HS, PE, and RA groups. Our results are like other reports suggesting that the percentage of Th17 cells increases when patients suffer an inflammatory condition such as PE or RA compared with HS (8,31). In this study, the PE/RA patients showed that the percentage of Th17 cells increased considerably and increased bone tissue destruction. PD and CAL determined periodontal tissue destruction and showed higher levels in patients with PE/RA. The percentage of Treg cells was similar in all groups. These results were consistent with previous reports suggesting that the percentages of Treg cells tended to decrease in PE and are similar in HS (32). The role of Tregs in RA is still unknown. In the present study, similar percentages were found in RA and RE/RA groups, and it may be due to the DMARDs treatment of RA patients. Studies have suggested that this therapy could influence Treg cells activation by decreasing their percentages. These drugs are prescribed to treat inflammation. Some studies have suggested that Treg increases with systemic inflammation, which does not occur in this population (33).

CONCLUSION

Despite its limitation, our study results suggest that PE in patients with RA increased the number of DNA copies of *P. gingivalis* in dental plaque, and it could be altering the immune response, increasing the percentages of Th17 cells, as well as the count and regulation of Treg cells. Further studies should confirm these findings and evaluate the damage that could cause IL-17

cytokine expression by Th17/Treg. It has been associated with bone destruction and resorption, clinical manifestations in both diseases.

FUNDING STATEMENT

None

CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization: R.E.M.M and J.L.A.H.
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Validation: C.A.M., L.F.E.C., R.A.D.P. and J.L.A.H.
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Supervision: J.L.A.H.

All authors have read and agreed to the published version of the manuscript.

ETHICAL CONSIDERATIONS

The study was approved by the Research Committee of the Master´s Degree Program in Advanced General Dentistry at the Autonomous University of San Luis Potosi, Mexico, Informed and voluntary written consent from patients was obtained by clinical examination according to the ethical principles of the Declaration of Helsinki.

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